Detection of Malaria Antibodies by means of Immunofluorescent Test using different stages of Parasites as Antigen obtained from In vitro Cultures and Human Subjects

DISSERTATION SUBMITTED
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BY
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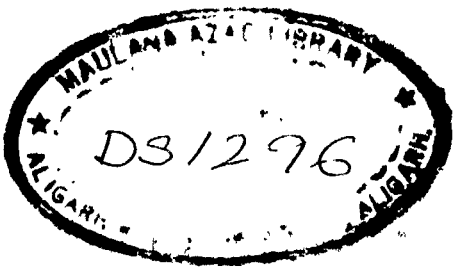

Dedicated to my
Parents and Brothers

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This is to certify that the work presented in this dissertation has been carried out by Mr. S. Farrukh Sultan Rizvi and is suitable for the award of the Master of Philosophy degree in Zoology of the Aligarh Muslim University, Aligarh.

ATHER H. SIDDIQI.

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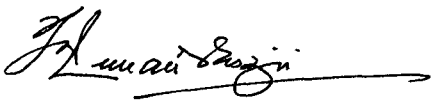


(S. FARRUKH SULTAN RIZVI)

ABSTRACT

Recent introduction of immunofluorescent techniques for sero diagnostic work in malaria has opened up immense possibilities for large scale seroepidemiological surveys in the endemic regions. The present investigation was designed to establish the utility of indirect fluorescent antibody (IFA) tests in the sero diagnosis of human malaria. The specific reactivity of these tests was evaluated in various categories of patients by employing slide impressions of the infected blood as the source of antigen.

Results indicate that IFA test using washed - cell thick smears is a sensitive, specific and reproducible laboratory procedure. A seropositivity rate of over 90% was obtainable by using schizonts as antigen model. Other blood stages were found to react less efficiently. The difference in reactivity was only quantitative having had little effect on the overall usefulness of the assay procedure.

Cross-reactions between different species of human malaria were somewhat more common. Using a 4-fold titer difference in homologous and heterologous species, identification of the infecting species was easily accomplished. The immune reactions in these tests were, however, not specific for various blood stages. The use of *in vitro* cultured parasites gave equally reliable results in these IFA tests. The results obtained on other serological tests such as ELISA and TIA. The IFA tests can therefore be successfully used for seroepidemiological purposes, and also as an adjunct to microscopic examination of the blood smears for establishing a reliable diagnosis especially in borderline cases of doubtful, wayside identification of malaria parasites.

CHAPTER I

INTRODUCTION

INTRODUCTION

The year Nineteen hundred and eighty marked the one hundredth anniversary of Laveran's discovery of the malarial parasite. Laveran's initial observations were from followed by extensive program of research on a global scale. Yet malaria continues as one of the world's most serious health problems. Over hundred million new cases occur each year, mostly in the developing countries of the world. In a large number of such endemic regions malaria may not be a direct cause of significant morbidity and mortality, but it acts as a "great umbrella" for making a large number of immune compromised individuals easily susceptible to other more life threatening diseases (Downs, 1975).

Malaria is caused by parasites belonging to the genus Plasmodium. The parasites infect a wide range of vertebrates. There are hundreds of well recognized parasite species, having different host specificities. All species require two hosts, a vertebrate and an invertebrate. The later being by definition, the definitive host.

Of the four species (P. falciparum, P. vivax, P. malariae and P. ovale) of Plasmodia infecting human beings, P. falciparum produces the most severe form of the disease. It has, therefore, deservingly received most of the attention of researchers and public health professionals. The disease produced by the other three species, though severe, produces few of the more acute symptoms commonly associated with P. falciparum infections.

Since the clinical picture presented by these species is highly variable, a need has been felt to develop alternate laboratory procedures for use as an adjunct to microscopic examination in which parasites may not

always be detectable. The demonstration of parasites in the blood of the host is considered diagnostic, though negative microscopic examinations do not always exclude the infection, altogether. This is particularly true in early infections when the parasite desnity is very low, or in cases where the blood picture is somewhat obliterated due to chemotherapy. In acute P. falciparum infections only rings are found in peripheral circulation, since the late stages invade the blood capillaries of the deeper tissues. The blood smear taken 2-3 hrs. after the onset of fever is generally negative, except in late infections where gametocytes may remain in circulation for quite a long time. For making a differential diagnosis it is often necessary to rely on indirect immunodiagnotic procedures, in addition to clinical observations.

The use of immunofluorescence techniques in the serodiagnosis of parasitic diseases has been steadily increasing over the last decade or so. Fluorescent antibody tests are now being successfully employed for large scale antibody monitoring, or immune surveillance. Infact, Immunofluorescent antibody (IFA) testing is a very useful technique far malarial serology. The procedure is simple and sensitive, needing a few easily available reagents. The IFA test is a two step method in which various dilutions of the serum are allowed to react with antigen on a thick or thin parasitized blood smear (Sulzer et al., 1969). After washing with phosphate buffer, a solution of fluorescein - labled anti IgG is poured on the slide for tagging the antigen-antibody complex. The slide is washed again and examined in ultraviolet light in a fluorescence microscope. The intensity of apple green fluorescence in is graded as a positive reaction. Serial dilutions of the test serum are used to establish a visual end point, the antibody titer being the last dilution yielding visible fluorescence. It is apparent that this method is subjective; but experienced workers are

able to obtain consistently reproducible results. The only difficulty sometimes encountered is in comparing results from other laboratories.

The provision of suitable malarial antigen has always been a limiting factor. Initial workers used thin blood smears from infected individuals or primates (Tobie and Coatney, 1961; Voller, 1962) as a source of antigen. Sulzer et al.,(1969) showed that thick smears prepared from washed infected cells could successfully be used even in cases of very low parasitaemia.

Homologous human plasmodia or closely related simian plasmodia were best used as antigen. This often poses some difficulties in obtaining good antigen source. The mature schizonts, which were known to be most reactive (Targett, 1970) do not occur in human peripheral circulation. This problem was somehow rectified with the descovery of Aotus monkeys, in which P. falciparum, P. vivax and P. malariae run a successful course of infection. Further, the development of long term in vitro culture of P. falciparum (Trager & Jensen, 1976) has to a certain extent solved this problem. Since the prepared slides can be stored at-70°C for several years, a few centralized facilities could supply all such antigen that might be required. But in all those laboratories where animal model and/or in vitro cultures facilities are lacking, the antigen is usually obtained only from infected persons.

Briefly, the objectives set out in the present investigations were:

(1) To evaluate the sensitivity and specificity of IFA tests using P. falciparum and P. vivax antigen.

(2) To assess the sensitivity and reactivity of different stages of parasites for use as a test antigen.

(3) To find out the test reactivity of early blood stages, as also of mature schizonts.

(4) To compare the IFA results with other serological methods such as, Enzyme-Linked Immunosorbant assay (ELISA) and Thin Layer Immunoassays (TIA). The last two tests have recently been used for serologic diagnosis of malaria in our laboratory.

CHAPTER II

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Malaria is one or the most wide spread disease in the world, affecting some 200 million people. In some regions, malaria transmission is so intense that the extent of present efforts at mosquitoe control and treatment of the disease have proved totally inadequate. In Africa alone, about one fourth of all adults suffer from malarial fever at one time or another. While other infected people have fever attacks due to the presence of a relative immunity or resistance. After the age of 12 months, almost every child in tropical Africa is reported to have malaria. About one million children every year are estimated to die (from malaria alone). In countries such as India and Sri Lanka, where malaria had regressed earlier, is is now resurgent.

The accepted name of the disease is malaria- the human infection caused by parasites belonging to genus Plasmodium. Although the name malaria derived from the Italian word for bad air does not appropriately signifies the cause of infection. It is a term which has become more common place, since the scientific term - plasmodioses- has never really come into wide use. Of the four species of Plasmodia infecting humans (P. falciparum, P. vivax, P. ovale and P. malariae), P. falciparum produces the most severe form of the disease.

A number of exhaustive reviews have been published by various WHO Expert committees on Malaria (1957, 1961, 1968b, 1972, 1973, 1974, 1977 and 1979). Wernsdorfer (1980) has also recently completed a very detailed literature review.

(A) IMPORTANCE OF MALARIA:

I. Historical:

Some early descriptions of a human disease like malaria

originating from ancient Egypt are found in Edwin Smith's surgical papyrus, 1600B.C. (Breasted, 1930). The papyrus Ebers, 1550 B.C. also refers to a similar condition associated with rigours and splenomegaly (Garnham, 1966).

The first accurate clinical discription of malarial fever was given by Hippocrates in 400 B.C. (Boyd, 1949) who mentioned the classic triad of chills, fever and sweating. He further analysed the characteristic periodicity of various forms of malaria and associated splenomegaly. He also described the endemicity of malaria and some of its topographic aspects. Celcius, in the first century A.D., gave a rather precise description of a febrile disease. He also described falciparum, vivax and quartan type of infections as some what separate entities. The earliest attempts to prevent what appears to have been malaria are contained in Edwin Smith's surgical papyrus (Breasted, 1930). There is a discussion on an oil from Balanite trees as a mosquitoe repellant (Garnham, 1966). The first attempt to an actual treatment of malarial fevers came in the middle of the seventeenth century with the introduction of the bark of a Peruvian tree. The bark was employed in local Indian medicine as a febrifuge, although its use was apparently quite limited (Jarcho, 1964). In 1820, Pelletier and caventon succeeded in extracting two alkaloids which they named quinine and cinchonine (Scott, 1939), out of which quinine was found more effective. Later, the use of pure quinine almost completely replaced the administration of cinchong bark. The administration of quinine was also subsequently replaced by synthetic antimalarials (Peters, 1980). The fortuitous discovery of cinchona was infact the only tool available for controlling the disease-which remained an enigma until the end of the nineteenth century. Advances in pathology, hematology, microbiology and parasitology, greatly aided by the development of better microscopes, brought malaria increasingly into the realm of research. The macroscop: pigmented appearance of the brain and spleen of patients who died due to malarial fever was known since the eighteenth century. But it was Meckel (1847) who observed black granules embedded in protoplasmic

In early part of the present century further work was carried out to investigate the various aspects of host-parasite relationship, life-cycle, pathology, chemotherapy and epidemiology. Such descriptions pertained to both human and non-human malarial parasites. Now-a-days the emphasis is mainly on the control of the disease because of the high morbidity and mortality. Due to the discovery of drug resistance in parasites and vectors, the researchers and medical health professionals are now working towards the development of a suitable vaccine (Siddiqui, 1979; Trager, 1979; Nussenzweig, 1980) against various stages of the parasites.

II. Present Malaria Situation:

At the beginning of 1979 about 2128 million persons were living in originally malarious areas of the world. This amounted to about 65% of the total world populations of 3287 million, China not included. Eradication operations have eliminated malaria from large parts of Americas and from Australia. In spite of a large scale use of insecticides and antimalarial drugs, the disease is already showing signs of a global come back.

In India the incidence dropped to an all time low of 60,000 cases in 1966 but reached a new peak of over 6.5 million in 1976. Although more than 90% of these infections were caused by *P. vivax*, yet a steadily increasing number of *P. falciparum* cases is a matter was nevertheless of serious concern. As a result of certain effective control measures, the number of cases actually dropped to 4.7 million in 1977 and 4.1 million in 1978. Although the data released by National Malaria Eradication Programme, Delhi (Table 1) on the malaria situation in India is somewhat bewildering.

In Aligarh district alone, the P. falciparum cases has now gone up from 76 in 1979 to 545 in 1981 (Table 2). In our laboratory, positive

TABLE I. *Showing blood smears collected, positives,* P. falciparum *and deaths from malaria in India from* 1965 *to* 1980

Year	Blood smears examined (in million)	Positives	P. falciparum	Deaths
1965	40.66	100185		
1966	39.83	148156	..	..
1967	40.42	278621	..	..
1968	41.99	274881	..	..
1969	41.84	348647	..	..
1970	40.96	694647	..	..
1971	40.45	1322288	..	..
1972	39.20	1428649	..	..
1973	42.45	1930272	..	..
1974	45.45	3167658	..	3
1975	51.82	5106142	729251	99
1976	55.98	6467215	753713	59
1977	67.01	4740900	451484	55
1978	60.46	4144385	548557	74
1979	61.42	3064697	558423	198
1980 (Provisional)	51.10	2206428	369596	146

Courtesy (NMEP)

TABLE 2

INCIDENCE OF MALARIA AT DISTRICT ALIGARH U.P.

Year	Blood Smear Examined.	POSITIVE BLOOD SMEARS				% Incidence
		P. vivax	P. falciparum	Mixed sp.	Total	
1977	228848	39224	1555	53	40832	17.8
1978	238531	32125	284	10	32419	13.5
1979	186055	7126	76	1	7203	3.8
1980	241929	5623	449	10	6082	2.5
1981	165717	6634	545	6	7185	4.3

cases for P. falciparum are showing a steady increase every year since 1980. From September 1982 to January 1982, this laboratory has recorded a 20 fold rise in falciparum cases.

III. Life Cycle:

The life cycles and morphology of human, non-human primates, rodents, avian and reptilian malarial parasites have been variously reviewed by Garnham (1966), Collins and Aikawa (1977), Carter and Diggs (1977), Seed and Manwell (1977) and Ayala (1977).

The sporozoites are inoculated into the blood circulation by an infected mosquitoe, from where they are transported to the host cells. Possibly, guided by chemotaxis, and recognizing its target, they enter into hepatocytes (as in mammalian plasmodia) or reticuloendothelial cells (as in avian plasmodia).

The observations made by Fairly(1945) in P. vivax infections indicated that sporozoites to enter the cells within 30 minutes after inoculation, growing to pre-erythrocytic schizonts after undergoing a series of morphological changes. The number of nuclear divisions and their interval vary widely from species to species (Wernsdorfer, 1980). After completion of the nuclear division, the cytoplasm segregates and merozoites are formed. In contrast to erythrocytic stages, pre-erythrocytic or exo-erythrocytic schizoints do not contain pigments. Conspicous round vacuoles were described in the exo-erythrocytic schzonts of P. vivax, while clefts, flocculi, or inclusions were reported in other plasmodia. Such morphological differences are typical of the species, permitting their identification. Plasmodium malariae, P. ovale and P. brasilianum are the only primate plasmodia in which the host cell nucleus is enlarged.

Erythrocytic merozoites are ovoid and possess an external membrane covered by a distinct surface coat of parasitic origin (Manson et al, 1977 The apical region contains the paired orgenelles (rhoptries) and a few micronemes which may be involved in the invasion of erythrocytes. During invasion of erythrocyte, the anterior end of a merozoite attached to the cell membrane, which after thickening forms a junction with the plasma membrane of the merozoite (Aikawa et al., 1978). The erythrocyte invaginate to form a parasitophorous vacuole in which the parasite eventually lies. The junction between erythrocytes membrane and plasma membrane of the merozoite assumes the forms of a ring which appears to travel along the merozoite surface. During this process of invasion the surface coat of the merozoite is lost, apparantly remaining outside the erythrocyte. Once within the parasitophorous vacuole, the parasite transforms rapidly into a young trophozoite which is finally surrounded by two membranes. The inner one is the original parasite membrane, and the outer one, which is contiguous with the exception of the cytosome area, is derived from the host cell. Mostly haemoglobin is ingested and is finally digested for form typical malarial pigment-hemozoin.

During the growth of the parasite, the amoeboid movement is well marked in younger trophozoites, but decreases when the trophozoite is fully grown, assuming a more or less rounded shape. The nuclear material increases and undergoes several divisions to give rise to presegmenter or preschizont stage. The cell containing fully differentiated merozoites is called a (mature) schizont. The mature schizonts burst and liberate individual merozoites, which in mammalian species can only invade new erythrocytes.

During schizogony, the infected erythrocytes may retain their normal size in P. falciparum and P. malariae or become enlarged as in P. vivax, or enlarged and deformed in P. ovale. Typical dots or

clefts may develop in the stroma of the infected red blood cells. The choice of red cells may be apparantly universal, as in P. falciparum or specific, e.g. Duffy-positive reticulocytes in P. vivax. The duration of schizogony is generally a multiple of 24 hours, usually 24, 48 or 72 hour. The mechanism of synchronicity observed in some species such as P. knowlesi and P. chabaudi are largely unknown (Collins and Aikawa, 1977).

Upon invading a new erythrocyte the merozoite can either initiate renewed blood schizogony or develop into a female or a male gametocyte. These gametocytes are taken up by mosquitoes where male gametocytes exflagellate. The exflagellated microgamete enters into a macrogamete and forms a zygote. Exflagellation is usually completed in 10-15 minutes. While the entry of microgamete in 1 minute (Sinden and Croll, 1975; Senden et al., 1978). These processes are separated by the time required for the microgamete to reach the macrogamete and to align itself. The zygote transforms into Ookinate which is actively motile (Speer et al., 1975) and invades into the host's intestinal epithelium. And through a series of developments "Oocysts and sporocysts containing numerous sporozoits are developed. The sporozoites reach the lumina of the silvary gland (Sterling et al., 1973) from where they are able to reach the vertebrate host with the next bite of the arthropod.

The life-cycles of all the four human plasmodia follow the same pattern showing slight variation in morphology, incubation period, number of divisions and pathology, etc.

(B) IN VITRO CULTIVATION OF MALARIAL PARASITE:

During the last few years the interest in the cultivation of all stages of malarial parasites has increased due to several reasons. Many current biological, biochemical, chemotherapeutic and immunological problems in malaria research can be answered adequately only by the use of reliable cultivation techniques. A successful culture

method inevitably supplies, information about the nutritional requirements of the parasite. At the same time it open up the possibilities of studying certain aspects which may not otherwise be possible within the body of a host. This is specially true of such parasites as the protozoa of human malaria, for which there are few suitable experimental animal models.

Culture of Erythrocytic Stages with in Their Host Cells:

(I) Short-Term Culture:

Following the first report of in vitro cultivations of P. falciparum and P. vivax by Bass and Johns (1912), little was done with short-term cultures until the work of Ball et al. (1945), and Geiman et al. (1946) with P. knowlesi. Later McGhee and Trager (1950) raised P. lophurae cultures. These workers were successful in being able to obtain complete development of intraerythrocytic stages of these parasites with some reinvasion and slight increases in parasite numbers. Essentially, these culture methods were more or less similar in their approach. These cultures are generally started with approximately 1% parasitemia established by mixing infected erythrocyte. with uninfected washed erythrocyts. The cells are then suspended in Harvard medium (Geiman et al., 1946) or one of the modifications of the Harvard medium (McGhee and Trager, 1950; Siddiqui and Schnell, 1972). The cultures grown in flasks or specially designed culture boats, both of these vessels provided a large surface to volume. The culture vessels were placed on a rocking platform (16 cycles/mm) and a gas mixture of 5% CO_2 and air was constantly passed through the vessels. Intra erythrocytic developmental stages of P. gallinaceum (Anderson, 1953), P. coatneyi (Trager, 1971) and P. falciparum (Siddiqui & Schnell, 1972) were obtained using similar short- term cultures. Such short term cultures could be used for experiments with chemotherapeutic agents (Black, 1946) and it is the basis of the in vitro test for chloroquine resistance developed by Riekmann and Antonano, 1971.

Plasma was used in all the short term cultures, although some attempts were made to replace it with other substances. Stearic acid, for instance, was found to support adequate parasite development (Siddiqui, 1977). The insistance on using plasma rather than serum in cultures was retrospectectively, a major obstacle in obtaining long term cultivation of erythrocytic stages of malarial parasites. The eventual use of serum was probably one of the most important steps towards the formulation of current methods of continuous cultivation of P. falciparum.

II. Long-term (continuous) Cultivation:

Until recently it has been impossible to sub-culture any species of malaria parasite through more than 3-4 cycles in vitro (Bertanga et al., 1972).

Trager (1976) and Trager & Jansen (1976) first reported the successful long-term culture of a chloroquine-resistant strain of P. falciparum from Aotus trivirgatus monkeys, using both a continuous flow system (Trager, 1971) and simple dilution system in petri dishes. The growth medium was RPMI-1640 supplemented with 25mM HEPES buffer, 0.2% (w/v) sodium bicarbonate and 10% (v/v) AB group human serum. The initial parasitized blood was diluted by the addition of normal human AB Rh+ type erythrocytes to give an initial parasite level of around 0.1-0.2% i.e. ca 10^6 parasites ml^{-1} and 10^9 rbc ml^{-1}. The medium was changed daily and a four fold multiplication in the parasite count was observed every 2nd day. The parasites retained normal morphology and were infective to Aotus monkeys. However, the parasites grown in vitro lost some of its synchronicity - a characteristic feature of P. falciparum, in vivo. Other strains have now been grown successfully in vitro in various laboratories as also in our laboratory.

Haynes (1976) has also described the laboratory technique for continuous cultivation of P. falciparum in vitro. He employed a more or less similar method, using medium-199 with 2-merapto - ethanol and ∝ - tocopherol.

Previous attempts to sub-culture erythrocytic stages of malarial parasites have been mainly restricted to P. falciparum and P. knowlesi. One such attempt was the cultivation of P. gallinaceum (Anderson, 1953) but the study has not been repeated. In all these cultures parasite dilution techniques similar to those described above, were employed. The parasites in such cultures survived for only three or four asexual cycles with a progressive decrease in multiplication at each cycle (Anfinsen et al. 1946; Trigg, 1969a; Trigg and Gutteridge, 1971; Phillips et al, 1972). There appears to be several important differences in the more recent work which may account for its success. How far these procedures are useful for sub-culturing other species is yet to be ascertained.

(i) Maintenance of pH;

Production of large amounts of lactic acid is one of the major problems encountered in in vitro cultivation. Lactic acid lowers the pH of the medium, thereby inhibiting the growth of the parasites. The maintenance of pH between 7.3 and 7.5 is critical (Geiman et al., 1966). Trager and Jensen (1976) and Haynes et al. (1976) have kept very low densities of parasites in their cultures, in order to reduce the lactic acid production. In addition, Zwitterionic buffers were employed to improve the buffering capacity of the medium. Earlier work by Geiman et al. (1966) had indicated an improved growth of P. knowlesi in medium containing 5 mM Zwitterionic buffer (glycylglycine; pka 7.9 at 37°C). However, it is better to use a zwitterionic buffer with a pka near to and slightly below the desired pH at 7.3-7.5. Trager and Jensen (1976) used 25 mM HEPES (pka 7.31 at 37°C) which theoretically appears to be the best buffer for these systems. Siddiqui and Schnell (1973) reported marginally better multiplication of P. falciparum

during one cycle *in vitro* using 40 mM TES (pka 7.16 at 37°C) rather than 40 mM HEPES. Haynes *et al.* (1976) successfully used TES in their system. In our laboratory we generally culture *P. falciparum in vitro* at pH 7.45. Zwitterionic buffers have further advantage of ensuring pH stability when cultures are manipulated outside the CO_2-rich atmosphere of the incubator. Cultures are also buffered with a bicarbonate/CO_2 system which requires adequate exchange between gaseous and liquid phases. A thin layer of cells covered by a shallow layer of medium (Trager and Jansen, 1976) is reported to improve the buffering. Trager (1971) found that the thickness of the cell and medium layer was critical for development of *P. falciparum in vitro*.

(ii) Susceptibility of Host Cells:

Recent work has indicated that the susceptibility of the host erythrocyte is an important factor. Initial experiments on the cultivation of *P. falciparum* from *Aotus* monkeys resulted in limited multiplication during a single *in vitro* cycle (Siddiqui *et al.*, 1970). Greater multiplication resulted by mixing parasitized blood from *Aotus* monkeys with human erythrocytes. Trager (1971) found that human group A, and not human group B or AB erythrocytes, agglutinates with *Aotus* erythrocytes. Trigg (1975), using a subculture technique showed that *P. falciparum* originally grown *in vitro* in *Aotus* erythrocytes would invade human group O cells to a greater extent than *Aotus* erythrocytes. However, Haynes *et al.* (1976) showed that *P. falciparum* invaded chimpanzee erythrocytes and as well as human erythrocytes (group not indicated). But these workers were unable to draw any conclusions as to the relative susceptibility of *Aotus* and human erythrocytes, since insufficient experiments were performed. The successful long term cultivation of *P. falciparum* was obtained in both instances, when the parasites were grown in human erythrocytes.

Reports so far available indicate that all the human ABO blood groups appear to be suitable. The significance of this was not clear since no one has reported attempts to cultivate P. falciparum serially in non-human erythrocytes. But it seems quite likely that human erythrocytes maintain their viability and/or susceptibility to infection in vitro better than non-human erythrocytes. Trigg and Shakespeare (1976a, b) have shown that normal Rhesus monkey erythrocytes incubated for periods upto 48 hr. in vitro are less susceptible to reinvasion by P. knowlesi as compared to unincubated erythrocytes obtained directly from the monkey. These observations may not contradict the finding by Trager and Jensen (1976) that successful cultivation of P. falciparum could be done in out dated blood from blood banks.

(iii) Removal of Leucocytes:

The successful use of out dated human blood for in vitro cultivation of P. falciparum may be linked with the reduction of leucocyte number. Bass and John (1912) stressed the necessity for removing leucocytes. But this aspect has been ignored by later workers, with the possible exception of Richards and Williams(1973), who removed the leucocytes by passing the blood through a column of CF 11 cellulose column. It is not known whether this technique results in an improved multiplication of parasites in vitro since no comparative studies are available. But it is interesting to note that in continuous cultivation of P. falciparum the leucocytes were removed either by aspiration (Trager and Jensen, 1976 ; Siddiqui et al., 1979) or by passage through a CF 11 cellulose column (Haynes et al., 1976).

(iv) Gas Phase:

The gas phase is an equally important factor, as medium and serum. High Oxygen concentrations are reported inhibitary. Although Oxygen appears to be essential, since complete maturation upto schizont stages of P. knowlesi does not occur under anaerobic conditions. (Trigg, 1969b) Earlier owork employed 20% O_2 (Taylor and Baker, 1968) but a reduction in Oxygen tension to 5% or below appeared to produce better multiplications (Trigg, 1969b; Butcher and Cohen, 1971; Trager and Jensen, 1976 and Siddiqui et al., 1979). Siddiqui and Colleague in all their cultures use speciality gas mixture containing 90% N_2; 8% CO_2 and 2% O_2. In our laboratory too we use siddiqui's speciality gas mixture.

Maintenance of cultures at low Oxygen tension is one method of maintening low redox potential in the cultures. Trigg (1969b) showe d that a redox potential of ca 140 mV and ca 170 mV was maintained in P. knowlesi cultures in 1% and 5% Oxygen respectively, as opposed to ca 230 mV in 20% Oxygen. That is the reason why Haynes et al(1976) use reducing agents like 2-mercaptoethanol and vitamin E (α-tocopherol in their med ium-199.

(v) Serum and Plasma:

Short term cultures have shown that plasma and sera from infected and normal monkeys vary considerably in their ability to support in vitro growth of P. knowlesi (Butcher and Cohen, 1971). Heat inactivated foetal calf serum also supported the growth of P. knowlesi (Trigg, 1973). Trager and Jensen (1980) routinely use A^+ serum but Siddiqui et al. (1979) use O^+ serum. Trager(1980) recommended the use of compatible serum with ABO type blood or

AB^+ serum for all new isolates. In our laboratary we use either A^+ or AB^+ serum. 10 to 15% serum supports the growth of P. falciparum quite well but levels significantly above 15% can have negative effects on growth. Human sera appear to vary in their ability to support growth. Commercially obtained sera are not suitable (Trager and Jensen 1980), neither is freshly collected fetal calf serum (Jensen, 1978b). New Zealand white rabbit serum is the only such serum identified to replace human serus (Siddiqui and Palmer, 1981).

Many attempts have been made to replace human serum by commercially available materials thereby eliminating the one major culture ingredient most difficult to obtain. Recently, Siddiqui (1979) reported use of 10% commercially available bovine serum supplemented with 5% red cell extracts to successfully replace human serum. Growth with this system was equivalent to that obtained with human serum but the requirement for the human red cell lysate proved to cause clumping in the cultures.

More recently, Siddiqui (1980) reported use of calf serum supplemented with 0.25% proteose peptone to totally replace human serum in continuous cultures. Growth and parasite morphology in these cultures is equal to that obtained with human serum.

The ability of serum to support growth varies only marginally. A strict check on the type of serum to be used is essential, especially in areas where malaria is endemic. Because immune serum and serum from individuals taking antimalarial drugs are detrimental to parasite growth and multiplication. It was recently shown that Indian sera samples even with low malaria antibody titres are not suitable for in vitro cultivation of P. falciparum (Rizvi and Ahmad, 1982). The parasites in cultures with Indian sera sample could complete one schizogonous cycle but were unable to invade fresh red cells. This effect was not noticeable in cultures containing sera from U.S. blood donors.

(vi) Culture Systems:

Trager and Jensen's original system utilizes plastic petri dishes containing 1.5 ml of an 8% red cell suspension. Infected cells are diluted so as to given an initial parasitaemia of 0.1-0.2%. Parasites are cultured in high CO_2 tension in petri dishes kept in dessicator at 37°C. Starting with a parasitaemia of 0.1% Trager and Jensen (1980) achieved a 20-50 fold increase to 2-5% parasitaemia after 96 hr.

Siddiqui (1979) utilized a flask type system instead of petri dish-candle jar method. These cultures are incubated in 125 ml Erlenmeyer flask containing 5% red cell suspension and a 1-2% starting parasitaemia. A constant supply of gas mixture, containing 2% O_2 8% CO_2 and balance nitrogen is made through the flasks. Using this system Siddiqui and Colleagues are able to obtain 20-30% parasitaemia with only daily medium change. In our laboratory we use the same system as that of Siddiqui and colleagues (1970). A major advantage of the flask system is that it can be adapted to a wide range of convenient volumes (Table 3).

More recently, Siddiqui and Colleagues (Ref.) have developed 12 litre Doms and Plexiglass box for large scale cultivations of P. falciparum. Further improvements in culture systems have also been done to automatic the repititive medium changes. These include the automated "Tripping-vessel" (Jensen et al., 1978) and computerized culture system (Siddiqui and Palmer, personal communication.

(C) IMMUNOASSAYS IN MALARIA:

I. Indirect Fluorescent Antibody (IFA) Test:

At it inception in early 1960's the IFA test was a novelty, and heroic efforts were required to obtain acceptable results. The

TABLE III

. OUTLINE OF *in Vitro* SYSTEM FOR *P. falciparum* SHOWING MODIFICATIONS FOR LARGE SCALE PRODUCTION[a]

Type of culture vessel	Total volume of culture (ml)	Packed erythrocytes type O[b] (ml)	RPMI-1640 medium (ml)	and	Human type O serum (ml)	or	RPMI-1640 medium with 0.25% proteose peptone (ml)	and	Calf serum (ml)
Microtiter plate (16 mm)	1	0.05	0.8		0.1		0.8		0.1
25-ml flask	2.5	0.125	2		0.25		2		0.25
125-ml flask	10	0.5	8		1		8		1
500-ml flask	20	1	16		2		16		2
2800-ml flask	100	5	—		—		80		10
12-liter dome	300	15	—		—		240		30
Plexiglas box	500	25	—		—		400		50

[a] All culture vessels are connected to a continuous flowing gas system (90% N_2, 8% CO_2, and 2% O_2).
[b] Reconstituted 1:1 with RPMI medium.

(Courtesy Prof. W. A. Siddiqui).

following decade and a half have seen a continuous improvement in equipment, reagents and availability of antigen.

Early investigators were obliged to use optical benches with annormal microscope at one end, carbon arc illumination at the other and the various solutions acting as filters in between. This contraption was very cumbersome and has fortunately been replaced by modern fluorescence microscopes which have a compact built in high intensity light source, carefully computed solid filters and special objectives some of which permit epiillumination. Most diagnostic laboratories now have such microscopes, so immunofluorescence can be carried out provided malarial antigen is available to such laboratories.

Fluorescen-labelled antiglobulins have also been much improved over the years. At first each laboratory was obliged to do its own conjugation, but now there are numerous antispecies conjugates such as FITC-anti-rabbit immuglobulin and well defined human immunoglobulin class-specific materials (fluoresceinlabelled anti human IgG; antihuman IgM etc.).

Although IFA tests have been performed widely for malarial antibody, it is difficult to make valid comparisons.

The results are generally expressed as titers. The values represent the last dilution showing visible fluorescence. It can be readily appreciated that this is a highly subjective value; it is influenced by the type of antigen, test conditions, reactivity of conjungates, microscopical system and offcourse, the observer. The use of reference antimalaral sera may permit a large measures of comparability among laboratories. Similarly, the use of objective measurements of fluorescence may also assist. However, such reference

antisera are not in general use, as also the quantitation of IFA method. Since these procedures are time consuming and requires sophisticated equipment (Manawadu and Voller, 1971; 1978ab), they have not been widely employed. Attempts to use absorbed soluble antigen with results read objectively on a fluorimeter (Sadun and Gore, 1968) gave promising results, though not in routine use. The recent improvement of solid phase immunofluorescence technique (FIAX-STIQ, I. D. T. Corporation) may lead to a resurgence of interest in objective immunofluorescence tests.

(i) Specificity of the Malaria IFA Test:

In the early work on rodent malaria, Voller (1962, 1965) noted that inspite of a fairly strong cross-reaction between P. berghei and P. vinckei, these species did not reach with a similar group of cross reacting primate malaria.

Voller (1962) in his IFA tests reported cross-reactions between different primate malarias, while Tobie et al., (1961) were of the opinion that differences in these results were quantitatives. They found that the titer was always highest in the homologous parasite-anti serum system. This difference between homologous and heterologous IFA system has been confirmed for many primate and human malarias (Collin et al., 1966a, b, 1967b; Diggs and Sadun, 1965; Wilson et al., 1970). Even though acknowledging such differences, Gartin et al., (1966) and Menwissen (1966, 1968) have suggested that P. cynomolgi, and the semian parasite P. fieldii, respectively, may be used as "all purpose malarial antigens, Sulzer et al., (1969), in their detailed comparison of human malarias by the IFA test, concluded that only human malarial parasite should be used for obtaining better results on these tests such tests in their opinion could be more useful if additional information on the probable species of the infecting parasite is also given. The data published by Diggs and Sadun (1965) further lend support to this view. Though a well documented study by Ludford et. al., (1972) doubts the validity

of the claims so far made on the specificity of IFA tests for malaria. They found extensive and high level cross-reactions between malaria and Babesia antigens. The sera from cattles having Babesia infection reacted strongly with P. falciparum antigen.

The multispecies malaria slide containing P. vivax, P. falciparum and P. malariae of Sulzer et. al., (1973) provides a suitable antigen for maximum sensitivity in detecting antibody to all human malarial infections. Unfortunately, the difficulties in obtaining such a preparation mean restrict its use to a very few centers.

(ii) Course of antibody production as measured by the Indirect Fluorescent Antibody test:

In one of the earliest publications on malarial IFAs Kuvin et. al., (1962) outlined the course of antibody production in volunteers infected with P. vivax or P. cynomolgi. The first appearance of antibody closely followed patent parasitaemia with titers rising rapidly over the next 2-3 weeks and declining thereafter. The titres tend to be at low levels after about 3 months. This was confirmed by Tobie et. al., (1960) who found that even in sporozoite induced infections, the antibody production begins only after the invasion of erythrocytes. Throughout the prepatent period no antibody could be detected. These authors mentioned that relapses again led to rapid rise in the antibody levels, but Lunn et. al., (1966) found that only some patients reacted this way. In others patients there was a transient decrease.

The earliest longitudinal antibody study on P. falciparum in non-immunes was made by Collins et. al., (1964b) Antibody levels were neither high nor varied considerably during the course of infection. However, reinoculation of semiimmunes resulted in antibody levels rapidly climbing to stable plateaus (Collins et. al., 1964c). Antibody could still be detected almost 2 years later in these patients.

Collins et. al., (1964a) also titrated antibody to P. malariae in induced P. malariae infections. In such cases, the antibody levels were higher and more stable than a P. falciparum infection. Lapascu et. al., (1966) extended the use of IFA in P. malariae infections, showing that the antibody response was related to the length and intensity of the infection. Reinoculation of P. malariae resulted in especially high titers.

All these early studies measured the total antibody response without making any distinction in the different classes of immunoglobulins making up the total response. As early as the mid-1960s, Abele et. al., (1965) and Tobie et. al., (1966) were able to show significant changes in IgM and IgG levels in induced malaria Collins et. al., (1971a) using immunoglobulin class-specific conjugates, measured the antibody content for its IgG, IgM and IgA levels. The IgM and IgA antibodies were transient showing that IgG antibodies were more persistent.

Targett (1970) also used class-specific conjugates in his study on P. falciparum in Gambia. The sera of residents there had significant levels of antibody in the IgG and IgM fractions with much less IgA contents, the IgE class was not detectable. Ambroise-Thomas et. al., (1971) studied a monkey with an induced P. cyanomolgi infection, and they too found early antibody of the IgM class but later all the antibodies were in the IgG fraction.

Fewer studies have been carried out on the serological response to rodent malarias using IFA tests. Voller (1965) reported a transient antibody response in rats infected with P. berghei. Waki and Suzuki (1974) found a similar response in P. berghei-infected mice with IgM levels declining rapidly. However, Cox et. al., (1969) and Cox and Turner (1970) noted that, with P. berghei Yoellii and P. vinckei in mice, the initial development of antibody was similar to that reported

by earlier workers but thereafter both IgG and IgM antibodies remained a plateau level.

(iii) Application in Nonendemic Area:

There is little value in the use of IFA tests for diagnosis of acute attacks of malarias. Conventional parasitological techniques are always useful in the detection of parasite in these cases. However, chronic malaria may be suspected in patients who have been in the tropics recently, having suggestive symptoms such as hepato-splenomegaly, etc. In these cases the serology can be more useful in confirming or ruling out malaria. The detection of malaria infection in blood donors is another obvious use. It is neither necessary nor practical to serum all donors for malaria in non-endemic areas, but high risk donors can be tested. Drauga et. al., (1969), using the IFA test, detected potential transfusion threats in P. malariae in an area that had been subjected to malaria eradication many years earlier. It has also been possible by the IFA method to identify the responsible donor following post transfusion-induced malaria (Fisher and Schultz, 1969; Bruce-Chwatt, 1974, Najem and Sulzer, 1976).

(iv) Applications in Areas where Malaria is or Has been Endemic:

The situation where serology, especially the IFA tests can be useful have been detailed in a World Health Organization (1974) publication. They are as follows:

(1) To establish malaria endemecity-especially age-specific indexes;

(2) To assess changes in transmission during or after antimalaria programs;

(3) To decrease malarious areas; and

(4) To identify areas or people requiring treatment for malaria, especially during control programs.

Voller and Bray (1962) first demonstrated that antibody could be detected in virtually all the inhabitants of malaria hyperendemic area in Liberia. They also showed that antibody levels increased with age. McGregor and Colleagues (1965) made a much more detailed study of the IFA test responders in Gambia, West Africa. Childrens borned with high levels of antibody showed a decline over their first few weeks of life. Thereafter the levels increased throughout childhood and during adult life. If pregnant women are given antimalarial protection and their newborn children maintained on malaria prophylaxis, they did not show antibodies detectable by IFA tests (Voller and Wilson, 1964). The work in Gambia and other early studies in North Africa (Coudert et. al., 1966) Nigeria (Voller and Bruce-Chwatt, 1968), and Malaysia (Collins et. al., 1968b) led to the realization that antibody profile of the population could be us ed as a measure of their malarial experience. This information could be of value in indicating actual malarial endenicity as well as changes following control measure (Collins et. al., 1968b; Leiijveld, 1971). led to the realization that antibody profile of the population could be used as a measure of their malarial experience. This information could be of value in indicating actual malarial endenicity as well as changes following control measure (Collins et. al., 1968b; Lelijveld, 1971). Some of the clearest results emerge from the East African work on the altitude delivation of malaria transmission. Both in Ethiopia (Collins et. al., 1971b) and Tanzania (Voller et., al., 1971) thrre was a clear difference in IFA response in people living above and below the critical altitude for transmission.

Draper and Voller (1972) published a mathematical model for interpreting age-related antibody prevalence rates, and it was

evaluated reasonably successfully in East Africa and Brazil. A much improved statistical method was later prepared by Van der Kaar (1975) and was validated in Surinam.

The precision of IFA test in identifying malarial foci has been confirmed by numerous publications of Edrissean and Afshar (1974) in Iran, Warran et. al. (1975) in central America, and Sulzer et. al., (1978) on their work in Peru. The serology made possible the localization of endemic foci in remote areas. Logzstic difficulties meant that blood slide examination would not have been feasible on the same scale.

The IFA test has been shown to reflect changes in malaria transmission. Bruce-Chwattand Draper (1973) and Bruce-Chwatt et. al., (1975) were able to show by IFA methods that malaria was no longer being transmitted in Maurituis and Greece, respectively. Similarly Ambroise-Thomas et. al., (1972) showed the absence of malaria in most of corsica following eradication activities, and the remaining transmission areas were readily identified.

Ambroise-Thomas and Colleagues (1976) also followed the progress of a large anti-malarial campaign in Tunisia. They found the IFA most useful when parasite rates were under 1%.

II. DIRECT METHOD:

In this method antimalarial immunoglobulin is labelled with a fluorescent dye such as fluorescein. This conjugate react with malaria parasites or antigen in blood smears or tissue sections. The method was used initially to show a wide antigenic cross-reactivity between different species of malarial parasites (Ingram et. al., 1961;

Tobie and Coatney, 1961; Voller, 1962). This method was of little value measure making antibodies, since each serum sample had to be individually labelled, requiring a large amount of serum. The method was also time-consuming.

The major use of such direct immunofluorescence method has been in immunopathological studies, particularly with respect to identification of various components of immune complexes on the kidney glomerular basement membrane in quartan malaria nephrotic syndrome. Allison et. al., (1969), ward and Kubukamusoke (1969), and Houba et. al., (1971) showed that in the nephrotic syndrom IgM and, less commonly, IgG and BIC were present in such complexes. About one third of the patients had malarial antigen in their complexes, and this was demonstrable by means of fluorescein-conjugated antisera to P. malariae. Voller et. al., (1973) also found IgM deposits in the glomerular of Aotus with induced quartan malaria infections. Similarly, direct immunofluorescence has been invaluable in studying the renal deposition of immunoglobulin, complement, and malarial antigen in P. berghei infections in Rodents (Ehrlich and Voller, 1972; Suzuki, 1974; Boonpucknavig et. al., 1972).

III. ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA):

Another indirect method for the detection of antimalarial antibodies is by means of ELISA tests. It is somewhat similar to IFA test except that an enzyme is used to label the antiglobulin instead of fluorescein. A colorimetric estimation of the enzyme by means of a chromogenic substrate replaces the visual, microscopical estimation of fluorescence in the IFA test.

In the indirect ELISA for malaria, soluble malarial antigen is passively absorbed to plastic test tubes or to the wells in micro-plates. The test serum is then reacted with the sensitized solid phase and incubated with an enzyme-tabelled antiglobulin after washing. After another washing step the enzyme substrate is added. Its color change can be estimated visually or photometrically and is proportional to the amount of antibody in the test serum.

(i) General Considerations:

(a) The Carrier Surface:

The carrier may be beads, tubes or plates. Some workers have used sepharose beads, since these permit covalent linkage of antigen or antibody to the surface (Deelder et. al., 1975). However, these test are less suitable for large scale use than the polystyrene tube method used by Engvall & Perlmann, (1971, 1972) or polystyrene micro-haemagglubination plates (Voller et. al., 1974.)

(b) Conjugates:

Engvall and Perlmann (1971, 1972) favoured alkaline phosphatase as an enzyme marker. It has high activity, and the chosen substrate is cheap and nontoxic, with a bright yellow reaction color that can be assessed visually or inexpensive micro ELISA readers. Conjugates of alkaline phosphatase can be stored at $4^{o}C$ with suitable preservative.

Peroxidase was shown long ago by Avrameas and Uriel (1966), Nakane and Purie (1966) and Nakane (1975) to be a good choice of enzyme for conjugation.

The choice of enzyme is largely a personal preference. The conjugates so far available are not entirely satisfactory, since they contain varying amounts of polymers and unconjugated proteins (Voller et. al., 1976).

(c) Test Parameters:

Certain other factors like incubation time, temp, antigen preparation vary from laboratory to laboratory. For convenience in our laboratory, plates are usually sensitized overnight at 4°C and serum and conjugate incubation time is 1 hour at 37°C. Shorter times tend to yield less accurate results;

(ii) Application of Indirect ELISA Tests:

Voller et. al. (1975) used ELISA with P. Knowlesi antigen, and they were able to measure malarial antibodies in human populations in Iran & Tanzania. However, the microplate method with P. falciparum antigen was more convenient and sensitive (Voller et. al., 1974). This latter method allowed identification of malarious areas in New Guinea, and usefulness of malaria control activities were reflected by obtaining lower ELISA values (Voller et. al., 1976). Recently this method was also used to follow antibody levels in Aotus monkeys infected with P. falciparum and it was found that such animals became ELISA-positive within a week of patent parasitaemia. But certain other studies using ELISA tests for measuring antibodies to P. knowlesi following vaccination showed that ELISA values do not accurately reflect protective immunity.

IV. Thin Layer Immuno Assay (TIA):

Thin layer immuno assay is a recently developed technique (Elwing et. al., 1976, 1977) which has been successfully applied to

the serodiagnosis of various parasitic infections (Nilsson et. al., 1980), as well as in herpes simplex viral infections (Jeansson et. al., 1979). TIA is characterized by extreme technical simplicity and high capacity, and is also suitable for screening large number of sera samples. These tests are carried out on polystyrene petri dishes coated with antigen. The antibody is applied to the holes punched in gel, layered on the plates. After allowing suitable time for diffusion, antigen-antibody reaction is visualized as condensation drops following exposure of plates to water vapours. The data of Nilsson et. al., (1980) indicated a good diagnostic specificity and sensitivity in TIA tests used for diagnosing filariasis, fascioliasis, schistosomasis and echinococcosis. However, they also noted most extensive cross reactions between echinococcossis and filariasis. The reasons for such cross reactivity reactions were however not elaborated. It is quite likely that the patients were infected with two different diseases, perhaps the two parasites may be sharing common antigens.

Recently Nilsson et. al., (1980) demonstrated the presence of antiamebic antibodies in patients with amoebic liver abcesses. The above finding attracted us to make use of TIA technique for malaria serology. Rizvi and Ahmad (1982) applied the TIA method for the detection of antimalarial antibodies employing in vitro cultured P. falciparum as antigen. The potential value of this test for diagnosisg. These results were comparable with those of enzyme-linked immunosorbant assays. However the TIA test results were not always reproducible, perhaps for lack of certain standardizations.

CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Media:

RPMI-1640 and Waymouth's MB-752/1 with L-glutamine and without sodium bicarbonate were purchased from Grand Island Biological Co., New York.

2. Sera:

(a) Human A, B, AB and O Rh^+ sera were obtained from Blood Banks of Hawaii, Honolulu, Hawaii and J.N. Medical College, A.M.U. Aligarh India.

(b) Foetal calf serum and calf serum was obtained from Grand Island Biological Co., New York.

3. HEPES Buffer:

HEPES buffer (N-2-Hydroxyethyl piperazine V-2-ethane sulphonic acid) pka = 7.5 at 25 °C and Mol. Wt. 238.3 was purchased from sigma (60F-5045) and Research Organic Inc. Cleveland, Ohio.

4. Antibiotics:

Injections of Gentamycin sulphate U.S.P., containing 40 mg gentamycin, 3.2 mg sodium bisulphate, 0.1 mg edatate disodium, 1.8 mg methyl paraben and 0.2 mg propylaparaben as preservatives, were used in all *in vitro* cultures. This was obtained from Wyeth Laboratories Inc., Philadelphia sodium azide (Riedel, Germany was used as preservative unless specified.

5. Conjugates:

(a) Antihuman Immunoglobulins - labelled with fluorecein isothiocyanate (FITC) was obtained from Wellcome Laboratories Beckenhan, U.K.

(b) Anti-human Immunoglobulins - Alkaline phosphatase conjugate in 0.05 M Tris (pH 8.0) containing 1% BSA, 1mM $MgCl_2$ and 0.02% NaN_3 and phosphatase substrate tables were purchased from Sigma Chemical Co., St. Lui.

6. Dye and Strains:

Leishman stain was obtained from B.D.H. England and Research Organic Inc. Cleveland. Coomassie brilliant blue was purchased fromB.D.H. - U.K.

7. Photographic Material:

Kodak Tri-X Pan 400 ASA was used for taking immuno-fluorescence photographs Rest of the photographs were taken on Kodacolor II 35 mm 100 ASA, Ektachrome 200 ASA and Kodachrome 64 ASA film. Al these films were obtained from Eastman Kodak Company, Rochester, NY through Kinsey Brothers, New Delhi.

8. Other Chemicals:

Sodium chloride, sodium dihydrogen phosphate, sodium barbitone, potassium hydrogen phosphate, sodium bicarbonate, sodium tetraborate, calcium Lactate, sodium acetate, sodium diethylbarbiturate, sodium hydroxide, Hydrochloric

acid etc. were purchased from BDH England. Ethanol and Methanol were obtained from Sarabhai Chemicals. Agarose was purchased from koch laboratories.

MAJOR EQUIPMENTS

1. Sterilization:

   (a) Laminar flow with0. 45 um HEPA filter model - 4 FTLA-FUNIT-237 manufactured by Klenzied contamination control valsad.

   (b) Autoclave model A25 - Ashok Vijay & Co., New Delhi.

2. Microscopy:

   (a) Visible Light

      (i) Zeiss Junior KF2 Mfd: Siemssen Inc., Germany

      (ii) Zeiss Amplival with Apochromate lenses, Carl Zeiss Jena, GDR.

   (b) Ultraviolet Light

      Bausch & Lomb Fluorescence illuminator with high pressure Osram 200 Watt bulb, Exciter filter S-58, density filter 2. 0 and eye cap barrier filters Y-8. Mfd. Rochester, N. Y. 14602.

3. pH-meter ECIL 401 with combination electrode.

4. K25 refrigerated centrifuge.

5. Gast Oil Pump, Philadelphia

6. Walk-in-incubator

7. Gas flow meters. gifted by the Deptt. of Tropical Medicine and Medical Microbiology, University of Hawaii.

EXPERIMENTAL

I. Washing of Glass Wares:

Pipettes were rinsed in a pipette washer for atleast four hours. The pipettes were then soaked in an acid solution containing conc. H_2SO_4, saturated aqueous $K_2Cr_2O_4$ and distilled water in 1:1:2 proportions. The pipettes were rinsed again in tap water and finally with distilled water.

All other glasswares were rinsed and immersed in acid solution. The glasswares were then rinsed free of acid and soaked in Teepol$^{(R)}$ (Sarabhai Chemical Co., India) overnight. The glass wares were then brushed and rinsed with distilled water for 4-5 times and dried.

II. Sterilization of Glasswares:

a. All glasswares prior to sterilization were washed with Teepol$^{(R)}$ detergent and thoroughly rinsed with tripple distilled water.

b. All washed glasswares were dried in hot air over.

c. All glasswares were plugged with gauge-cotton and rapped in aluminium foils.

d. The glasswares were autoclaved at 121^{o}C and 15 Lb inch^{-2} for 30 mts.

e. All pipettes were also sterilized in separate containers.

III. Collection of Infected Erythrocytes:

(A) Examination of Slides:

(a) Leishman Stain

150 mg of powder stain was dissolved in 100 ml methanol and kept in incubator at 37°C for 48 hrs. and filtered for use.

(b) Diluting Buffer

Na_2HPO_4	1.0 gm
KH_2PO_4	0.7 gm

Q.S. to 1000 ml with distilled water.

Protocol:

1. Thick and thin blood smears of patients attending OPD were prepared on precleaned slides in our laboratory.

2. Thin blood smears were fixed in methanol for 4 minutes and dried, using hot air blower.

3. Smears were then covered with Leishman stain (diluted 1:1 C buffer) for 30 minutes.

4. Slides were washed gently with PBS, dried and examined under microscope for malarial parasite.

(B) Isolation of Infected Blood Samples:

(i) Acid Citrate Dextrose (A. C. D.)

(a)

Citirc Acid	8. 0 gm
Sodium citrate	22. 0 gm
Glucose	24. 5 gm

Q. S. to 1000 ml distilled water

(b) Citrate Phosphate Dextrose (CPD)

Citric Acid	3. 27 gm
Sodium Citrate	26. 3 gm
Glucose	25. 5 gm

Sodium monobasic phosphate 2. 22 gm.

Q. S. to 1000 ml with distilled water

(ii) Protocol:

* Blood was collected from patients showing malarial parasites in smears.

* 2-5 ml blood was drawn, with plastipek sterile disposible syringes, in tubes containing ACD or CPD at a conc. of 15 ml ACD or CPD per 100 ml blood.

* Anticoagulant and blood was mixed thoroughly and kept at $4^{o}C$ until use.

* Anticoagulant was sterilized through 0. 45 Um millipore filter prior to blood collection.

IV. SET UP FOR IN VITRO CULTIVATION OF Plasmodium falciparum:

(i) Materials:

1. Erlenmeyer, fernbach or other culture flasks.

2. Black rubber stopper with two holes to fit in the culture flasks.

3. Glass tubing or cut pasteur pipets-cut to fit in the above stoppers.

4. Latex hose to fit in the glass tubing.

5. An erlenmyer flask used as a bubbler flask at the end of each gas line.

6. A gas washing bottle for placing on the gas line between the gas tank and culture vessels for humidify the gas system.

7. Gas flow meters to control the rate of gas flow.

8. Miscellaneous pipets, and other glassware properly washed and sterilized before use.

(ii) Reagents:

A. Medium:

Medium was prepared by dissolving one package of RPMI-1640 powdered medium with L-glutamine and without sodium bicarbonate (GIBCO, Grand Island,

New York 14072, Cat. No. 430-1800) in approximately 950 ml tripple distilled water 5.95 gm of HEPES and 2.0 gm of sodium bicarbonate was added to the medium. pH was adjusted to 7.45 using ECIL digital pH-meter. The medium was filtered through 0.45Um millipore filter. 0.625 ml of gentamycin (40 mg/ml) was added prior to filtration. The medium was aliquoted into small, screw, capped sterile tubes until used.

B. Proteose Peptone Stock Solution:

15 gm of Proteose Peptone (DIFCO) was dissolved in 100 ml distilled water with constant stirring and heat, and aliquoted into small tubes. This solution was autoclaved at 15 lb/inch^{-2} for 10 minutes and kept at 4^{o}C until needed.

C. RPMI medium with Proteose Peptone:

0.25 ml medium with Proteose Peptone Stock solution was added to each 100 ml of medium before use.

(iii) CULTURE METHODS:

A. Preparation of Normal RBC's:

1. Freshly collected or out dated cells were used for in vitro cultivation of P. falciparum.

2. The cells were aliquoted into small screw capped tubes and centrifuged at 1000 RPM for 10 minutes at 4^{o}C in REMI K-25 refrige rated centrifuge.

3. Supernatant alongwith buffy coat was removed carefully with pasteur pipet using Gast oil suction pump at 20 mm of Hg.

4. Equal volumes of RPMI-1640 medium was mixed with packed erythrocytes and again centrifuged at 1000 RPM for 10 minutes.

5. The cells were washed 5 times by resuspending them in RPMI-1640 medium.

6. After final wash the erythrocytes were diluted 1:1 with RPMI-1640 medium and stored at 4°C until needed.

B. Preparation of infected Cells:

1. The infected red blood cells were collected in anticoagulants described elsewhere.

2. The cells were washed 5 times with RPMI-1640 medium as above.

3. A drop of cell suspension was spread over a coverslip.

4. Coverslips were fixed, stained and the parasite count made in 10^4 erythrocytes.

5. The cells were mixed with normal erythrocytes so as to give an initial parasitaemia of 1-2%.

(iv) Culture Protocol:

Our culture system is based on the method described by Siddiqui et. al., 1979. The cultures were held in 125 ml erlenmyer flasks. A total 10 ml volume contained 10% red cell suspension (5% packed cell), 10% human serum and 90% medium. We routinely used A Rh^+ or

O Rh^+ red cells with compatible human serum. A 0.25% Protose peptone was used when calf serum was replaced with human serum in all adapted cultures.

A. 1 ml erythrocytes containing infected and normal cells were put into a 125 ml erlenmyer flask.

B. 8.0 ml RPMI-1640 medium and 1 ml serum was added to the flasks.

C. All manipulatious were carried out in sterile environment under Laminar flow hood.

D. On day O, the cultures were so adjusted as to have a 1-2% starting parasitaemia.

E. On day 1, the medium was not changed.

F. From day 2 the medium was changed daily. This was done by carefully aspirating the medium larger without disturbing the settled layer of erythrocytes.

G. A small sample was routinely obtained from the c culture flask making total and differential parasite counts.

H. Medium change was daily carried out by adding fresh 8.0 ml medium and 1 ml serum.

I. The flasks were then swirled gently to resuspend the settled layer after which the flasks were placed back in 37°C incubator.

J. All the flasks were connected together by amber latex tubing.

K. Humidified gas mixture containing 2% O_2 8% CO_2 and balance nitrogen was kept constantly flowing through the entire system at the rate of approximately 10 ml/minute^{-1}.

(v) Harvest and Sub-culture:

A. Subcultures were regularly made when parasitaemia reached 10-15% or on the 7th day which ever was earlier.

B. Infected cells were washed with RPMI-1640 medium and samples were stained to count parasite density.

C. On the basis of the count the volume of infected material was calaculated so as to be added to fresh RBC's for making up a total volume of 1 ml with a parasitaemia of 1-2 per cent.

D. Calculated volumes of infected material were dispensed in a number of flasks.

E. To each flask fresh diluted RBC's were added to make up to 1 ml volume.

F. Zero hour sample was prepared.

G. 8 ml RPMI-1640 medium with 1 ml serum was added to each flask.

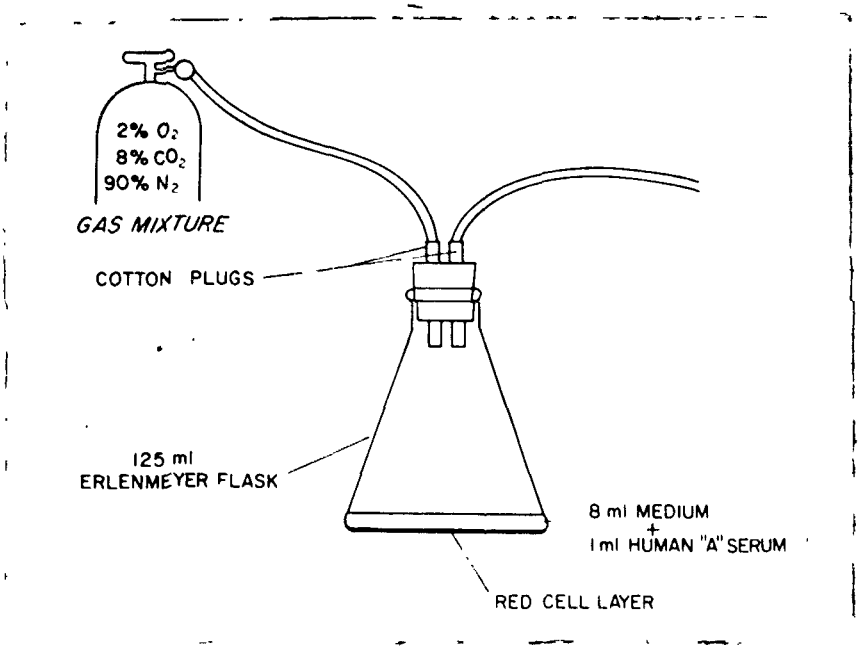


Fig. 1. A simple culture system for in vitro propagation of Plasmodium falciparum. (Courtesy Prof. W. A. Siddiqui).

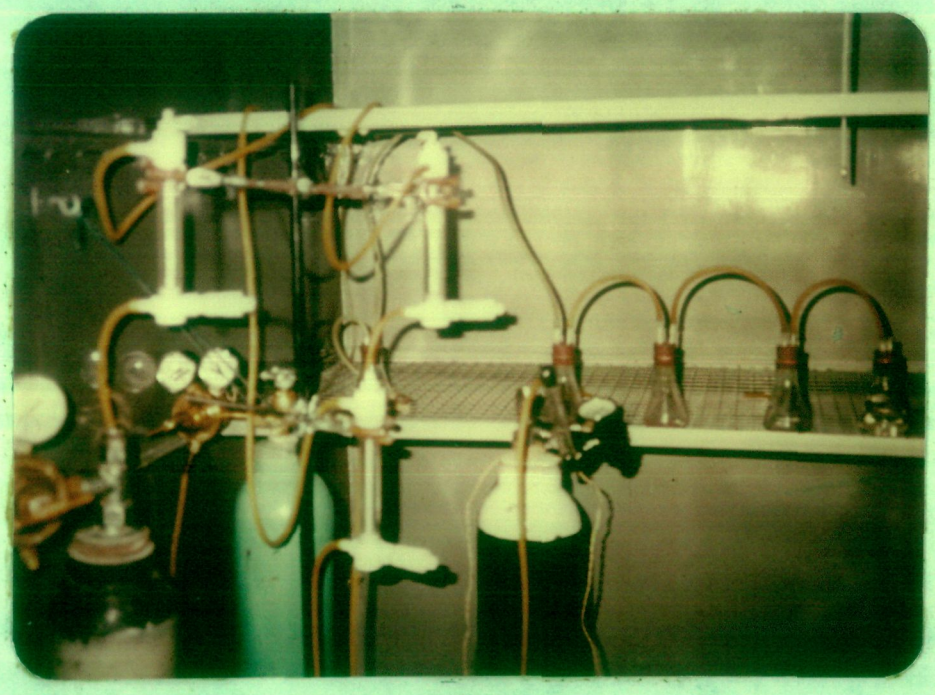


Fig. 2. *In vitro* culture set up inside walk-in incubator.

H. The flasks were incubated as usual iwith gas supply.

I. If antigen was required then only. on sub-culture flask was started which the rest of the material was pooled to isolate the parasites.

V. SHORT TERM IN VITRO CULTIVATION OF P. VIVAX.

It has not been possible to culture P. vivax on continuous basis. However, short term cultures were initiated to obtain the parasitic material. Infected blood was washed with medium and incubated as such in 125 ml erlenmyer flasks. Samples were taken every 12 hours. The parasites were harvested when all the blood stages matured to schizont stages.

VI. PREPARATION OF P. falciparum ANTIGEN:

Antigen was prepared from in vitro culture pools of P. falciparum for use in ELISA, CIE and TIE tests, etc. Since these cultures were asynchronous the Parasitized cells were harvested from different flasks in which parasites were mostly in trophozoite stage, or in late schizont stages. Both crude and partially purified antigen preparations were used in immuno-assays to be described later. The parasitized cells were given certain preliminary treatments before their use an antigen. The laboratory procedures used were as follows:.

(A) Preparation of Kreb's Glucose Saline:

Sodium chloride	6.9 gm
Potassium chloride	0.35 gm
Calcium chloride	1.28 gm
Pottassium biphosphate	0.16 gm
Magnesium sulphate	0.29 gm
Sodium bicarbonate	2.10 gm
Glucose	1.16 gm

Q.S. to 1000 ml with distilled water and filtered.

(B) Preparation of Stock Saponin Solution:

1 gm of Saponin was completely dissolved in 75 ml of KGS and filtered through Whatman No. 1 filter paper.

(C) Preparation of Discontinuous Sucrose Density Gradient:

Discontinuous sucrose density gradient was prepared in a centrifuge tube. The three layered gradient contained 37% lower layer, 23% middle layer and 10% topmost layer. All the three layers used were of equal volumes (Siddiqui et. al., 1978a).

(D) Harvest of parasitized Cells:

The cultures were monitoried daily using Leishman stained blood films. The cultures were checked until the maximum number of schizonts with fully matured merozoites were found. The supernatant was aspirated using vaccum pump under Laminar flow cabinet. The cells with remaining medium were poured into a narrow tube and centrifuged at 1250 g for 20 minutes. The upper dark layer containing

parasitized cells and few leukocytes was removed and washed twice with KGS.

(E) Preparation of Crude whole parasite antigen:

The two antigen preparations used in various immuno-assays were prepared as follows:

The washed and parasitized cells were resuspended in equal volumes of KGS and sonicated for 1 minute. The lysate was then centrifuged at 10,000 RPM for 30 mts. and supernatant was stored at -20°C. The washed infected cells were suspended in 20 times their volume in 1% (v/v) of stock saponin solution (1ml stock saponin in 99 ml KGS). This suspension was kept for 30 minutes at room temperature. The parasites were pelleted by centrifugation. The pellets containing intact parasites, few leukocytes and cellular debris were washed twice in KGS and were diluted 1:1 with KGS. This suspension was sonicated and the supernatant stored as described above .

(F) Partial purification of antigen:

Ssmples of partially purified P. falciparum antigen used in these studies were obtained from the department of Tropical Medicine and Medical Microbiology, Leahi Hospital, Honolulu, Hawaii. Few preparations were also made in our laboratory as follows:

The saponin treated pellets were diluted 1:5 with KGS and layered on top of the discontinuous sucrose density gradient with the sample volume being approximately one third the volume on the gradient. The loaded gradient was centrifuged at 750 g for 7 minutes at 4°C (Williamson et. al., 1975). Two to three bands were usually obtained after centrifugation. The upper brownish band contained the parasites

within the 10% portion of the sucrose gradient. This layer was carefully removed with the help of pasteur pipette. The parasites were washed with KGS and sonicated as described earlier.

(G) PREPARATION OF P. VIVAX ANTIGEN:

Since it was not possible to culture P. vivax for a long time in vitro, the blood samples obtained from patients were incubated as such for 30-40 hrs in culture falsks described earlier. If the blood sample already contained schizonts, then these cells were as such used for antigen preparation.

The washed-infected cells were subjected to all such treatments as for P. falciparum before they were used as antigen.

VII. INDIRECT FLUORESCENT ANTIBODY (IFA) TEST:

(i) Preparation of Phosphate Buffer saline, pH 7.2

Na_2HPO_4	1.0 gm
KH_2PO_4	0.7 gm
NaCl	8.5 gm

Q.S. to 1000 ml (W/V) with distilled water and filtered through millipore filtered.

Preparation of Blood Smear Antigen for IFA:

Both tick and thin blood smears were prepared from infected blood obtained from human patients as well as in vitro culture. The smears prepared from human patients infected with P. falciparum contained only rings, since mature forms are rarely encountered in peripheral circulation. The antigen smears with mature forms of parasites were prepared from

short term in vitro culture for(P. vivax) and long term cultures (for P. falciparum).

(ii) Ring stages antigen smears:

Infected blood was obtained only from those in which blood smears were positive cases of malaria. The blood was drawn in tubes containing ACD or CPD and washed 5 x C RPMI-1640 medium washed cells were reconstituted with RPMI-1640 medium, AB Rh^+ type human serum or calf serum. A drop of this blood was spread on coverslips. Such coverslips were fixed for 10 minutes in cold acetone and stored at -20°C until ready for use.

(iii) Mature stage antigen smears:

The cultures (described elsewhere) were monitored daily using Leishman-stained blood films. The cultures were checked till maximum number of schizonts with fully developed merozoites were found. The smears were prepared every 12-18 hrs to get different developmental stages. At the end of 40-48 hrs incubation, the blood was centrifuged at low speed (400 g) to increase the schizont density. The upper brown layer was used to prepare smears containing fully mature schizonts.

(iv) IMMUNOFLUORESCENCE STAINING PROTOCOL:

1. Packets of coverslips were removed from the freezer and allowed to stay at room temperature before uncovering.

2. Each coverslip was divided in half by first scoring with a dimond pencil.

3. Slides were numbered at back, using a water proof marker. An area of approximately 1 cm in diameter was marked where cells were evenly distributed.

4. Two fold serial dilutions of serum were made using PBS pH 7.2.

5. 0.5 ml of diluted serum was added to circled areas on each coverslip.

6. Coverslips were incubated from 30 minutes in moist chambers at 37°C.

7. Coverslips were washed gently with buffer to remove serum and replaced in moist chamber.

8. Circled areas were then covered with 0.05 ml of conjugate (FITC-Anti IgG) while the coverslips were reincubated for 30 minutes at 37°C in moist chamber.

9. Coverslips were washed with buffer and allowed to stand for 15 minutes with changing of buffer several times.

10. Excess moisture was removed with a Kim Wipe and coverslips were mounted in 50% buffered glycerol.

(v) SCORING OF SLIDES:

Slides were examined under fluorescence microscope (Bausch & Lomb) with 5-50 UV excitor filter.

Slides were scored as:

4 + *bright fluorescence*
3 + moderate fluorescence
2 + moderate fluorescence
+ weak fluorescence
± hard to tell; probably negative
- No fluorescence.

Positive slides were photographed using SLR photomicrographic assembly on Kodak Tri X 400 ASA film.

VIII. ENZYME LINKED IMMUNOSORBANT ASSY (ELISA):

(I) Preparation of Reagents:

(a) Coating buffer (0.05M Carbonate buffer, pH 9.6):

Na_2CO_3	1.59 gm
$NaHCO_3$	2.93 gm
NaN_3	0.20 gm

Q.S. to one litre with distilled water and stored at 4°C for not more than 2 weeks.

(b) PBS-Tween PH 7.4:

NaCl	8.0 gm
KH_2PO_4	0.2 gm
Na_2HPO_4	2.9 gm
Tween 20	0.5 ml

Q.S. to one litre with distilled water and stored at 4°C.

(c) Diethanolamine buffer pH 9.8

Diethanolamine	97 ml
$MgCl_2.6H_2O$	100 mg
NaN_3	0.2 gm
Distilled water	800 ml

1M HCl was added until the pH was 9.8

(II) Conjugates:

Alkaline phosphatase - labled sheep anti-human IgG(Sigma) was diluted 1:1000 with PBS-Tween pH 7.4 immediately before use.

(III) Substrate:

One p-nitrophenyl phosphate table (5 mg) was dissolved in 5 ml diethanolamine buffer which was warmed to room temperature and kept in dark until used.

(IV) PROTOCOL:

1. 200 Ul of soluble antigen, diluted with 0.05 M carbonate buffer pH 9.6, was added to each well of a disposable polystyrene microhemagglutination plate (Dynatech M29 AR Micro ELISA plates).

2. The plates were kept overnight at 4°C.

3. Antigen solution was removed by washing the plates three times with PBS-Tween for 3-5 mts.

4. The plates were vigorously shaken, inverted and drained on paper towels to get rid of excess fluid.

5. 200 Ul of test plasma or serum diluted with PBS-Tween was then added to the wells and incubated 1 hr at 37°C. The plates were again washed.

6. 200 Ul of diluted conjugate was added to each well, and plates incubated for 1 hr at 37°C and washed several times.

7. 20 Ul of enzyme substrate was added to each well and incubated for 30 minutes at 37°C.

8. The reaction was stopped with 50 Ul of 3M NaOH.

9. Positive results were shown by coloured wells.

10. Calf serum and PBS were used as serum and antigen control respectively.

11. For quantitative estimation of titers values, serial serum dilution were used.

12. Last serum dilution showing visible color was taken as the highest litre.

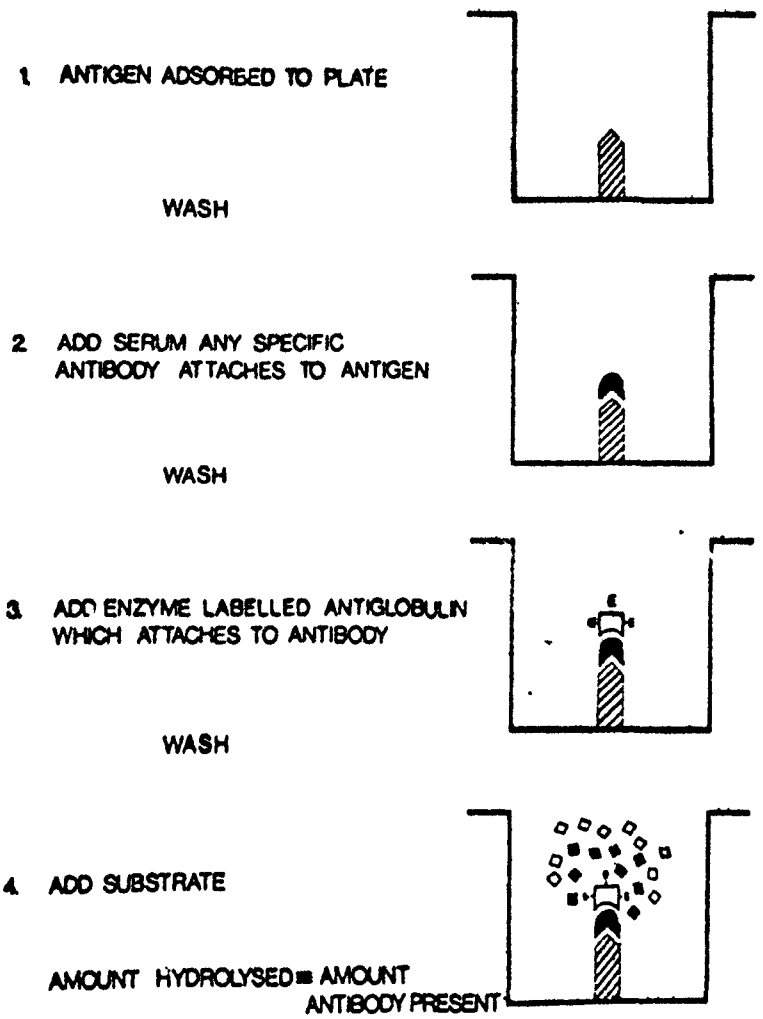
13. The ELISA tests were also carried out at different temperature, buffers and incubation time. The protocol of two different experiments is summarized in table 4.

TABLE 4

EXPERIMENTAL PROTOCOL OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

	EXP. I	EXP. II
Antigen	Whole parasite (sonicated)	Whole parasite (sonicated)
Buffer	PBS (0. 01 mol/liter)	Carbonate/bicarbonate (0. 05 mol/liter)
pH	7. 2	9. 6
Time	3 hrs.	Overnight
Temperature	37°C	4°C
Washing fluid	PBS+0. 2% Tween 20	PBS+0. 05% Tween 20
Serum dilutions	PBS+0. 2% Tween 20	PBS+0. 05% Tween 20
Incubation time	8 hrs	2 hrs
Temperature	Room	37°C
Conjugate dilution	1:2000	1:1000
Buffer	PBS	PBS+0. 05% Tween 20
Incubation time & temp.	2 hr at 37°C	1 hr at room temp.
Enzyme reaction	Alkaline phosphatase + p-nitrophenyl phosphate	Alkaline phosphatase + p-nitrophenyl phosphate
Time	1 hr	30 min
Temperature	Room	37°C
Stop	NaOH	NaOH

FIGURE 4
The Indirect ELISA for measuring antibody



Voller et al., 1976

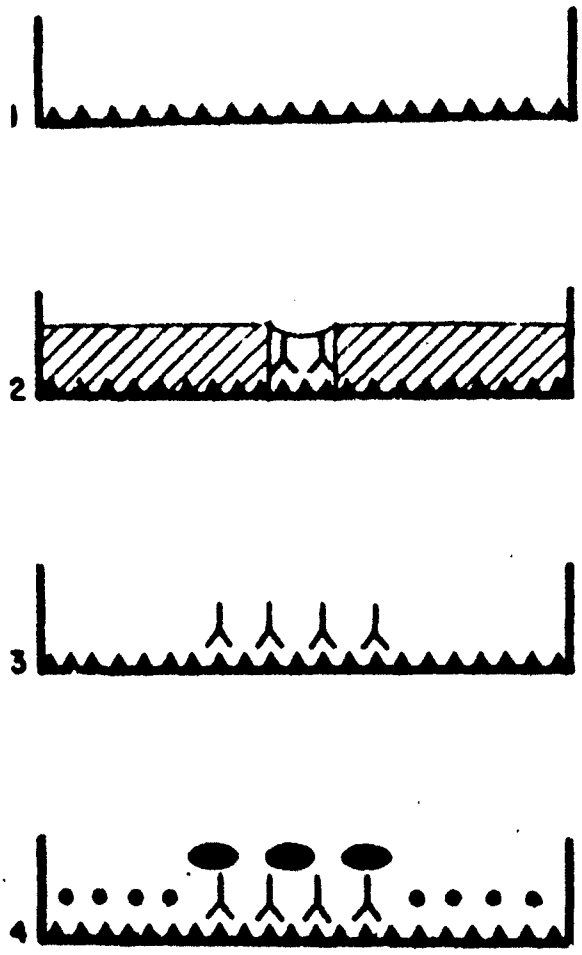


Fig. 5. Principle of DIG-TIA (Elwing et al., 1976)

IX. THIN LAYER IMMUNOASSAY (TIA)

TIA was performed by diffusion - in - gel modification technique described by Elwing et. al., 1976. A Schematic illustration of the principles of the technique is given in fig. 5. Polysterene petri dishes (8.5 cm, steriware) were coated with whole parasite antigen (described elsewhere) for 1 hour at room temperature. The plates were rinsed with distilled water and were filled with 1% Agarose containing 1% Foetal calf serum to form a 2 mm thick gel layer. About 10 to 15 circular holes (3 mm diameter) were punched in congealed gel with the help of gel puncher. The wells were filled with approximately 20 Ul of the test serum samples. After diffusion for 48 hrs at room temperature, the gel was removed from the plates.

The plates were rinsed once with distilled water and were flooded with a 1:500 dilution of anti-human - IgG for 1 hr. This was done to reinforce the antigen - antibody reactions. After washing, the surface was dried and possible antigen antibody reactions were visualized by exposure of the plates to water vapours at 56^{O} for 60 seconds. Zone diameters of the hydrophobic condensation drops were recorded.

CHAPTER IV

RESULTS

RESULTS

1. IN VITRO CULTIVATION OF P. falciparum:

The parasite yields in stationary flask system are summarised in table 5. The distribution of intraerythrocytic asexual stages of the parasite during continuous in vitro cultivation of 4 strains are given in table 5. The parasitaemia on day 0 was approximately 1-2% while on day 4 the parasitaemia ranged between 5.6-11.9%. There was an average increase of 4-5 fold in parasitaemia for each 4-day period with an approximately 10-fold per week net increase. On day 0 the erythrocyte suspension was 5% and at the end of 4 days it usually droped to approximately 3%. Due to frequent loss of red cells at each transfer, the peak parasitaemia usually remained at 10-15%.

In all the cultures the parasites were seen to go through a successful schizgonous cycle maturing to schizont stages. As is evident from the results, the population of parasites in vitro culture system mostly consisted of rings and trophozoite forms. Schizonts and segmenters constituted only 30-40% of the parasite population at the end of 96 hour. The number of merozoites usually averaged to 12-24 per segmenter.

(a) Effects of various concentrations of serum on in vitro growth of P. falciparum:

Routinely about 10% human serum in regular cultures and 15% in the new isolates is employed. The results of few isolates cultured in vitro in different concentrations of human serum are given in table 6. The extent of multiplication at the end of day 3 in 10 and 15% serum was higher than at 5% serum concentration. However

TABLE 5

Typical distribution of Asexual Stages during in vitro cultivation of Plasmodium falciparum.

Isolate	Incubation' hrs.	Initial parasitaemia per too r b c	Differential Count*					Increase
			R	T	S_2	> S_2	G	
F-25-I	0	16	3	6	5	1	1	
	48	47	16	12	90	6	3	
	96	56	10	8	23	12	1	4x
F-25-II	0	19	2	4	5	6	2	
	48	60	43	5	5	5	2	
	96	72	5	2	41	25	1	3x
F-22	0	23	10	4	4	5	1	
	48	81	48	15	3	15		
	96	119	15	40	32	24		5x
F-I	0	27	7	2	10	8	1	
	48	59	10	15	11	22		
	96	67	25	15	17	7		3x

* R, ring ; T, trophozoite ; S_2, 2 nucleated schizont ; > S_2, more than 2 nucleated schizont.

' Cultures were incubated at 37°C, gased with 2% 0_2, 8% Co_2 & 90% N_2.

TABLE 6

Effect of Different Concentrations of Human Serum on the growth and multiplication of Plasmodium falciparum in vitro

Isolate	Flask'	Serum conc.	Initial parasits per 1000 rbc	Extent of multiplication*			
				0-1	1-2	2-3	0-3
F-25-I	1	5%	13	2.1	1.2	1.4	2.0
	2	10%	10	3.1	1.4	4.6	9.8
	3	15%	15	3.3	1.2	2.6	6.4
F-22 (a)	1	10%	20	1.7	2.4	2.5	3.8
	2	15%	18	1.4	1.7	1.6	2.1
F-25-II	1	10%	14	1.7	3.5	1.9	4.6
	2	15%	17	1.4	5.0	1.7	2.6
FAG-1	1	10%	20	2.4	1.7	2.2	6.9
	2	15%	18	2.8	3.3	1.2	4.9

* Ratio of count on later day to count on earlier days.

' Total volume of flask was 10 ml containing 5% red cell suspension in RPMI-1640 medium containing 25 m M HEPES.

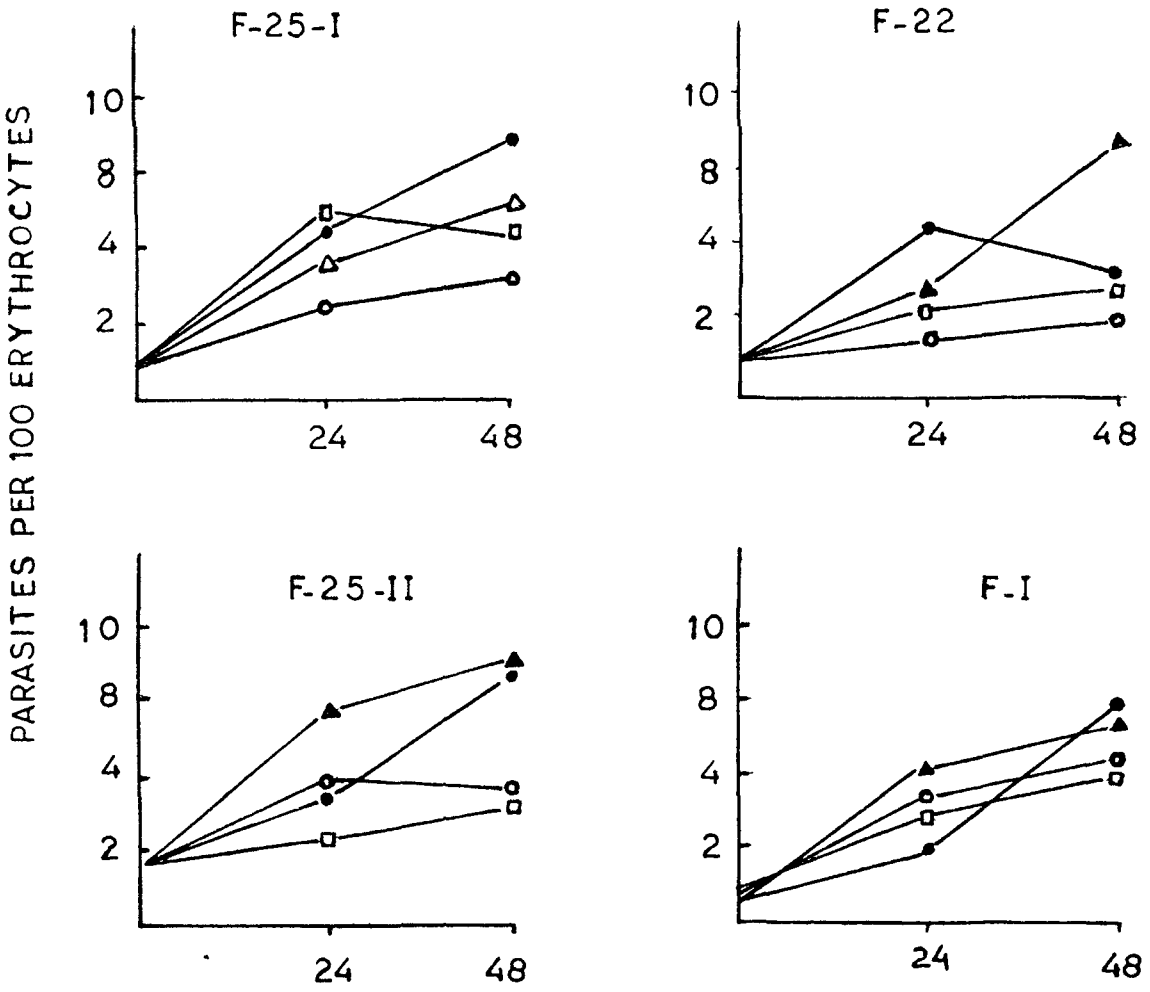
the extent of multiplication of an isolate of F-22(a) at 15% serum, the ratio of multiplication on day 1, 2 and 3 remained more or less constant as there was no change in the overall parasite count at the end of day 3. In the other three isolates no significant change in the overall growth was observed. In 5% serum, the parasite were found to complete one schizogonous cycle but further reinvasion was inhibited. The parasite morphology in such cultures was not normal perfectly. Both the chromatin and cytoplasm were found somewhat damaged in a number of parasites. A definite crination of the infected cell surfaces was also observed.

(b) Infectivity of Various cell types:

In most of the cultures the A^+ or O^+ red cells with compatible or AB^+ serum was employed. The growth pattern of 4 isolates in A^+, B^+, AB^+ and O^+ erythrocytes is shown in Fig. III. There was no appreciable difference in the susceptibility of different cell types. The infectivity of a cell infact, varied from strain to strain and also from person to person. However, the multiplication and reinvasion of P. falciparum was better in A^+ and O^+ erythrocytes.

(c) Effect of human serum on the growth of P. falciparum:

Generally the use of compatible serum with cell types or AB^+ serum in our cultures was found necessary. These sera were obtained from the Honolulu blood bank, Hawaii. No systematic study on the use of various serum types was attempted. But use of the Indian sera from the blood banks of J. N. Medical College A. M. U. Aligarh, in the cultures generally did not give very satisfactory results. The comparative results obtained in the sera from two different places are given in table 7. The parasites grown in Indian sera samples were seen to complete their schizogonous cycle at the end of 48 h.



COMPARATIVE STUDIES ON THE SUSCEPTIBILITY OF 4 STRAINS TO DIFFERENT ERYTHROCYTE TYPES in vitro

A^+ B^+ O^+ AB^+

FIGURE III

TABLE 7

In vitro cultivation of *Plasmodium falciparum* using sera samples obtained from Indian Blood Donors and U.S. Blood Donors.

Serum Source	Incubation time (hr.)	Parasites per 1000 R B C	Differential stages of parasites*					
			R	T	S_2	$>S_2$	G	Extracellular & degenerated
INDIAN	0	18	2	4	6	5	1	-
BLOOD	48	160	43	5	5	5	2	6
DONORS	72	69	24	39	5	1	-	8
	96	57	6	21	8	22	-	4
U.S.	0	23	10	4	4	5	-	2
BLOOD	48	56	10	22	10	15	-	2
DONORS	72	81	48	15	3	15	-	3
	96	119	19	48	24	24	-	2

* R, ring ; T, trophozoites ; S_2, 2-nucleated schizont ; $>S_2$, segmenter
G, gametocytes.

with little or no reinvasion. Most of the parasites were developed only upto the trophozoite stage and appeared to degenerate later. On the other hand the parasites in the serum obtained from U.S. blood donors were found to support the parasite growth for prolonged periods, the peak parasitaemia at the end of 96 hour in such cultures was 11-15%. The parasite density in cultures with serum obtained from Indian blood donors was never beyond 4-5%. The population of extra-cellular and degenerated parasites was comparatively much more in these cultures. Stages other than trophozoite were mostly abnormal both in the morphology and staining characteristics. Many erythrocytes were seen with merozoites sticking to the cell membrane as also the infected cells were generally seen to form clumps. Various developmental stages of P. falciparum and P. vivax. grown in vitro are shown in figures 6 to 12.

2. IN VITRO CULTIVATION OF Plasmodium vivax:

Several attempts were also made to culture P. vivax using in a system similar to that of P. falciparum. The results of a preliminary experiment are summerized in table 8. The majority of the parasites in a flask containing RPMI-1640 medium at 0 hr. were in the ring and early trophozoite stages. At the end of the 24 hr. incubation about 45% of the parasite developed to mature schizonts but at the end of 48 hr incubation, though a new generation of rings was found, the total number of parasites almost always decreased, with a corresponding increase in the degenerated and as well as extra-celluar parasites. Multiplication of parasites was better in flask containing waymouth's MB-752/1 medium. About 90% of the parasites were able to mature but there was no reinvasion whatsoever. The total number of parasites at the end of 48 hr. incubation remained the same as at the start of the experiment soon after only a very few viable parasites were seen in the culture. The culturing P. vivax was not successful beyond 5 to 6 days.

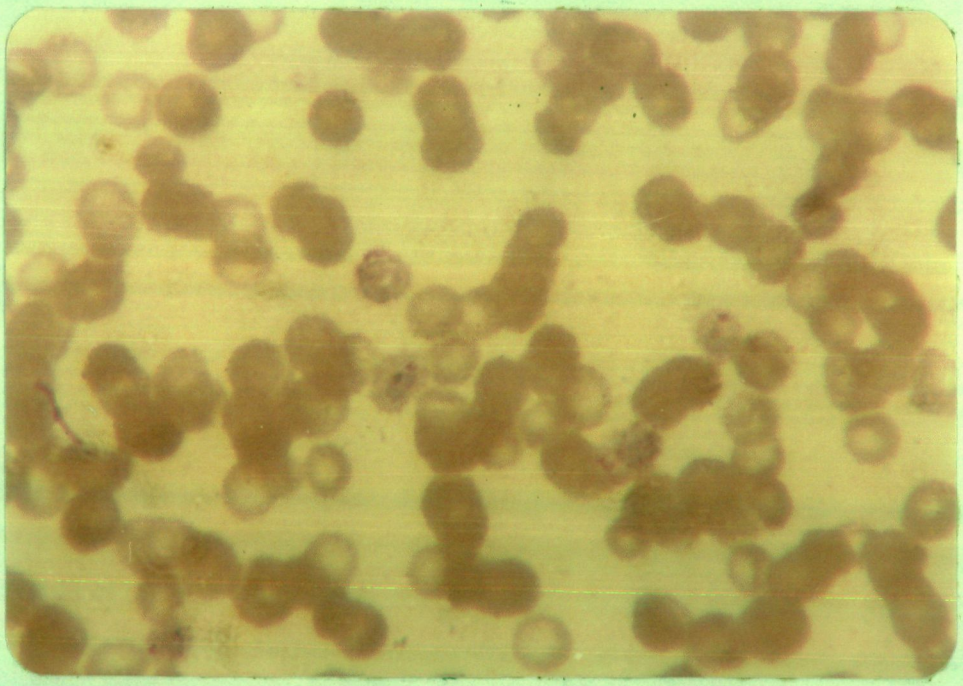


Fig. 6. Showing Plasmodium vivax trophozoites from a culture flask.

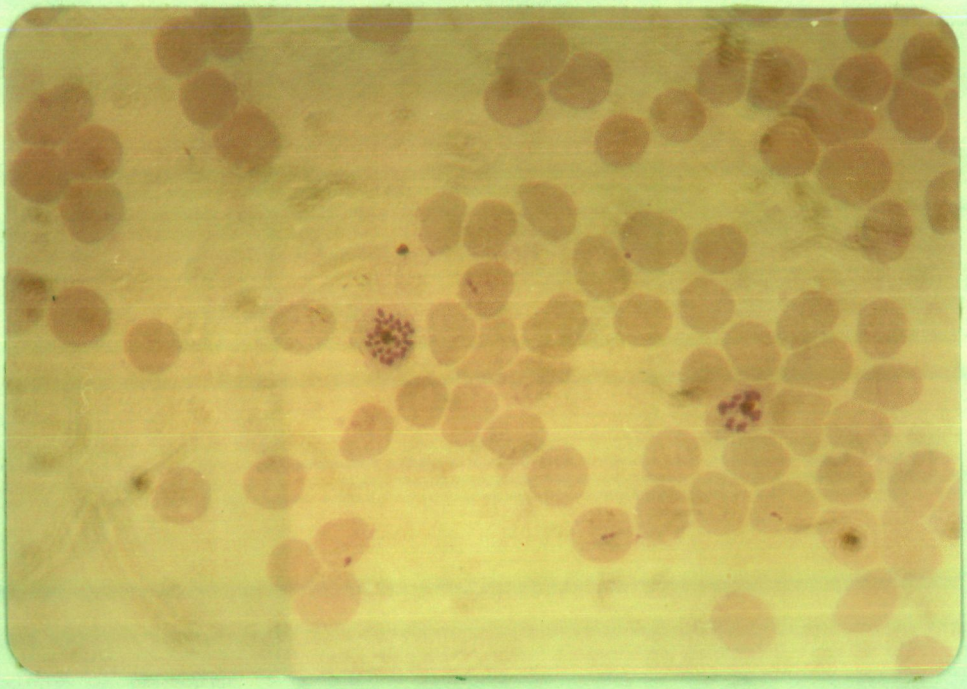


Fig. 7. Mature schizonts of P. vivax from a culture flask.

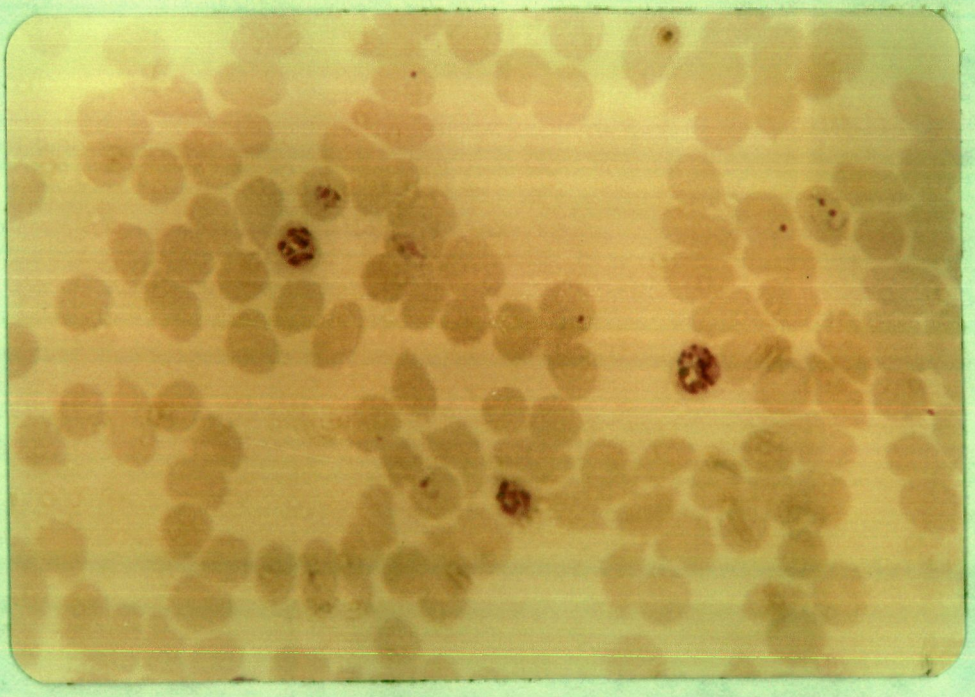


Fig. 8. Ring and schizont stages of Plasmodium falciparum from a culture flask.

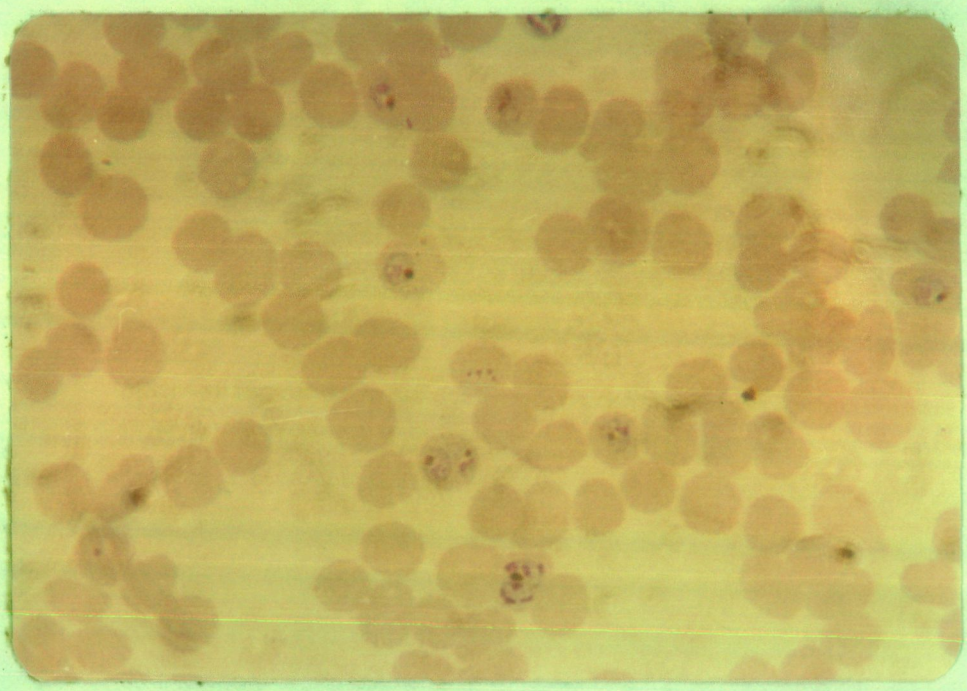


Fig. 9. Mature trophozoites and 2-nucleated schizonts of P. falciparum after 24 hrs. of incubation in an in vitro culture.

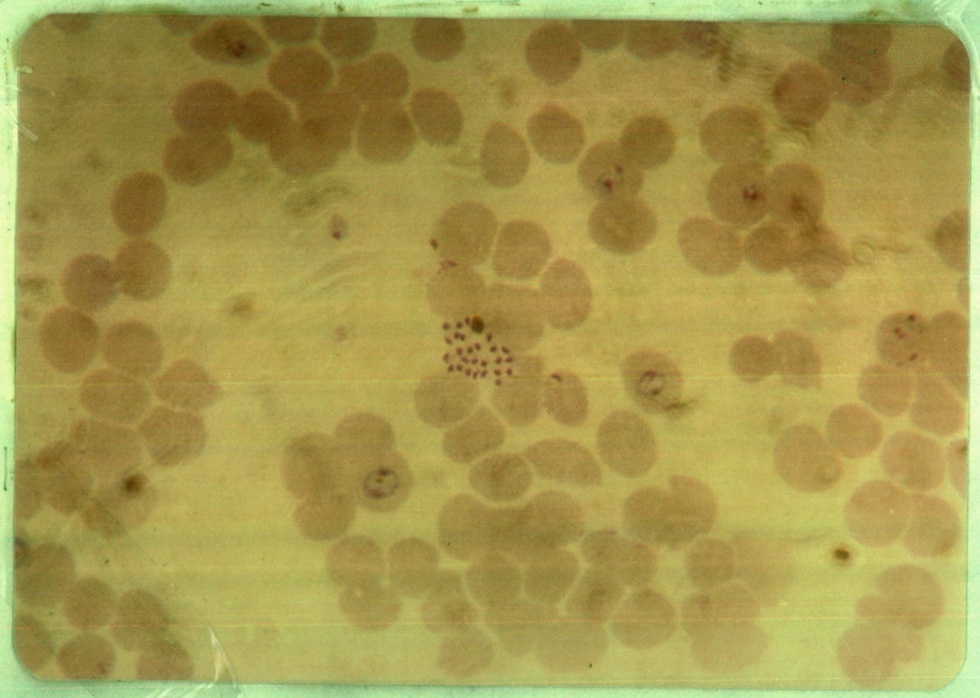


Fig. 10. Shows a fully mature segmenter of P. falciparum with individual merozoites. Haemozoin pegment is also seen in the center.

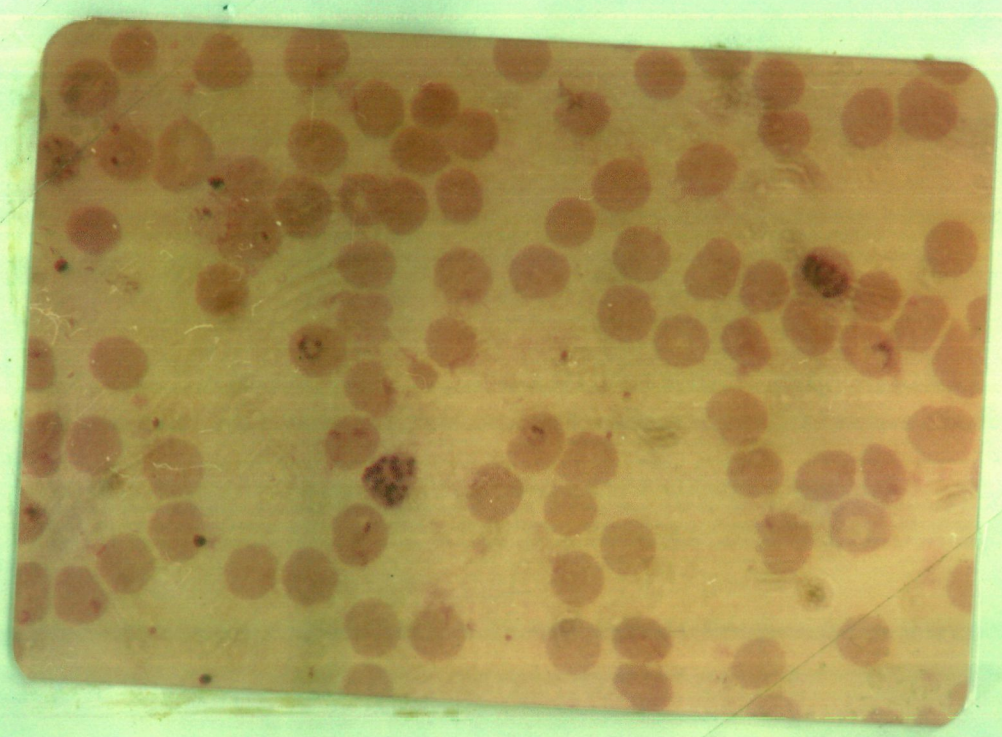


Fig. 11. Showing another fully matured schizont at 48 hr incubation. The reinvasion of RBC by a few rings can also be seen.

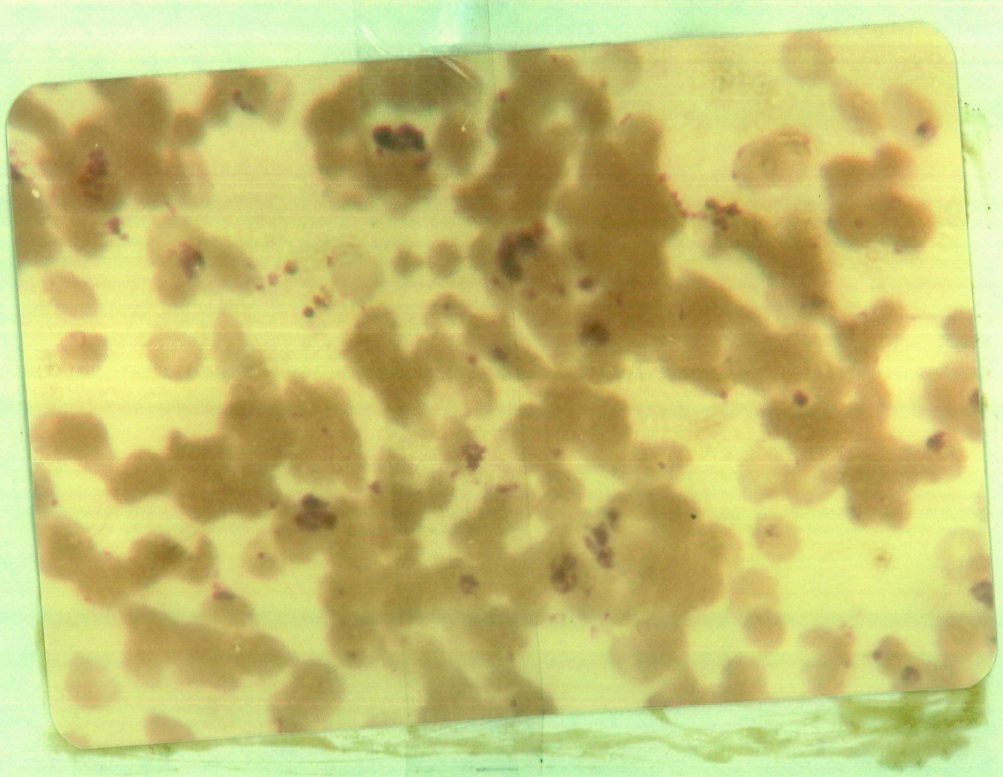


Fig. 12. A slide smear of P. falciparum from a culture flask showing approximately 10% parasitaemia.

TABLE 8

In vitro cultivation of Plasmodium vivax

Medium	Incubation time (hr.)	Parasites per 1000 R B C	Differential stages of parasites*					
			R	T	S_2	$> S_2$	G	Ext. & deg.
RPMI-1640	0	12	8	2	1	1	-	-
	24	19	-	2	8	9	-	2
	48	8	2	2	2	2	-	4
Waymouth's MB-752/1	0	13	6	4	-	3	-	1
	24	21	1	2	8	10	-	2
	48	10	4	4	2	-	-	8

* R, ring ; T, trophozoite ; S_2, 2-nucleated schizont ; $> S2$, more than 2 nucleated schizont and segmenter ; G, gametocyte ; ext & deg. extracellular & degenerated parasites.

3. INDIRECT FLUORESCENT ANTIBODY TEST FOR MALARIA:

Washed-cells on a thick smear were used as the source of antigen for all IFA tests. The test set up included about 15-20 sera samples at a times. The data presented here represents the test results obtained on the various sera samples.

A total of 165 sera samples were tested including 12 from suspected cases of fever. The diagnosis on the rest of them was confirmed by microscopic examination of the blood smear. In separate study sera samples from normal populations were also included. The distribution of IFA titer in 153 cases of malaria is shown in table 9. Only 5 (3.2%) of the samples showed negative finding. The overall specificity of the test was found greater than 95%. False positive reactions were obtained in less than 3% cases. A titer of 1:16 was considered to be the cut off titer between negative and positive results. All sera samples below 1:16 titer were considered as negative for IFA test. Sera samples with both P. falciparum and P. vivax antigen were tested for assaying the reactivity and the specificity of the IFA tests. Table 10 shows the distribution of IFA titres in the two types of sera. One of the tests included sera from 0-14 years of age group, a while the other group included sera taken from individuals of 15 years and above. Six samples in the first (0-14 years) category and 11 of the second category (16 years and above) were IFA positive, though the blood smears from these individuals were negative microscopically. The average titres were recorded at around 1:32 or less. The sera samples taken during the course of an active disease and in the post treatment periods were also tested. In both the instances the IFA test sensitivity was about 95%. The percent reactivity of the IFA tests is shown in table 11. The antigen used for such tests is generally obtained from in vitro

TABLE 9

IFAT on the sera samples obtained from malaria and suspected fever cases.

Species	Total samples Examined.	Number of sera with end point titer of							
		Neg.'	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
P. falciparum	65	2	4	4	18	10	15	8	4
P. vivax	85	3	3	18	28	20	8	3	2
Mixed Species	3	0	0	1	2	-	-	-	-
Suspected fever cases	12*	8	1	2	1	-	-	-	-

' Titers 16 were considered as negative.

* include 2 cases of Primary Syphils and 2 cases of chronic tuberculosis.

TABLE 10

IFA tests on the sera obtained from residents of Gabhana.

Age group (Years)	Parasitological results.	No. of person examined	No. of sera with IFA titer of						
			16	16	32	64	128	256	512
0-14	Positive	25	4	1	1	8	9	1	1
	Negative	10	4	4	-	2	-	-	-
	Total	35	8	5	1	10	9	1	1
>15	Positive	65	8	10	15	-	24	4	4
	Negative	21	9	5	6	-	-	-	1
	Total	86	17	15	21	-	24	4	5

TABLE 11

Reactivity of washed-cell thick smear antigen in IFA tests.

Sera group	IFA titers	Washed-cell thick smear antigen of P. falciparum No.	P. falciparum %	P. vivax No.	P. vivax %
Acute infection	Negative	6	14.5	8	16.0
	1:16	2	5.5	2	4.0
	1:32	24	60.0	20	40.0
	1:64	8	20.0	20	40.0
Post treatment	Negative	6	12.2	2	2.3
	1:16	10	21.4	2	2.3
	1:32	33	64.4	8	95.4
	1:64	1	2.0	0	0.0

TABLE 12

Comparative evaluation of the reactivity of different stages of plasmodial antigens in IFA tests using anti sera with an end point titer of 1:128.

Serum sample#	Reciprocal IFA titer with thick smear antigen of Trophozoites			
	Rings	Intact	Dehemoglobinized	Gametocytes
PF-02.1	-	1:16	1:32	1:32
PF-02.4	-	1:32	1:64	1:32
PF-25.4	-	1:64	1:64	-
PF-05.2	1:8	1.128	1:128	-
PF-07.2	1:8	1:16	1:64	1:64
PF-25.2	1:8	1:32	1:128	1:64
PV-09.1	-	1:32	1:64	-
PV-12.2	-	1:64	1:128	-
PV-19.4	-	1:32	1:32	-
PV-49.0	-	1:64	1:64	1:32
Total replicates	2	5	5	2

PF, P. falciparum ; PV, P. vivax ; IFA, Indirect Fluorescent Antibody test.

cultivation pools but antigen-smear slides were also prepared directly from infected individuals whenever possible. Most of the false positive results were seen when antigen smear obtained directly from the patients were used. Such results were less common when washed-cells smears from cultures were employed. The washing of parasitized cells generally removes soluble serum components, especially the gamma globulin that may contain malaria antibodies. This was sometimes found to alter the IFA test results. In table 11 most of the sera were previously screened by other serologic tests and were found to have low antibody concentration. Sera of low antibody concentration were mostly chosen for the IFA tests, in order to check the reactivity of the tests procedure. The sera with high antibody titer are infact readily detectable by other serological tests.

The reproducibility of the test titres with thick smear antigen on a test-to-test basis was found excellent (table 9). Four positive sero were tested with each of the two antigen speacies several times. All the titers were found to replicate with in plus or minus one dilution factor in a four fold serial dilution.

Figure 13-20 shows the photomicrographs of the indirect fluorescent antibody (IFA) tests. After a series of procedural manipulations the test slides were visualized under the UV light in a fluorescent microscope. The parasites showed an apple-green fluorescence in a dark back ground. With careful precision it was possible to see the size, shape and some of the morphological features of the infected cells. The reactivity of the different stages of the parasites in thick-smear antigen slides was thus assayed in these IFA tests. Sera samples giving antibody titers of 1:64 or more in other serological tests were included in this test. Both intact and dehaemoglobinized

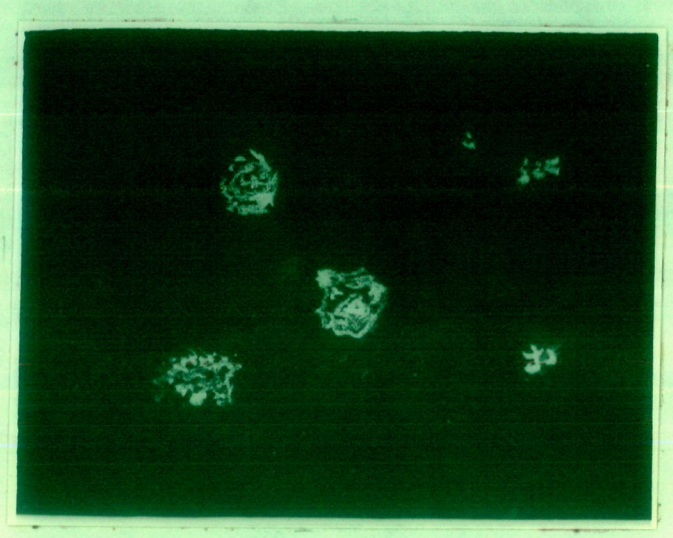


Fig. 13. Visual impression of 4^+ fluorescence of a trophozoite of Plasmodium vivax.

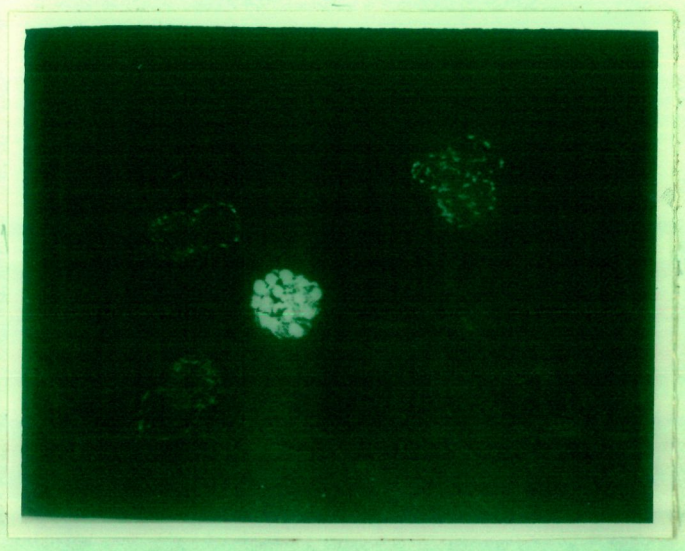


Fig. 14. Visual impression of 4^+ fluorescence of a mature schizont of P. vivax.

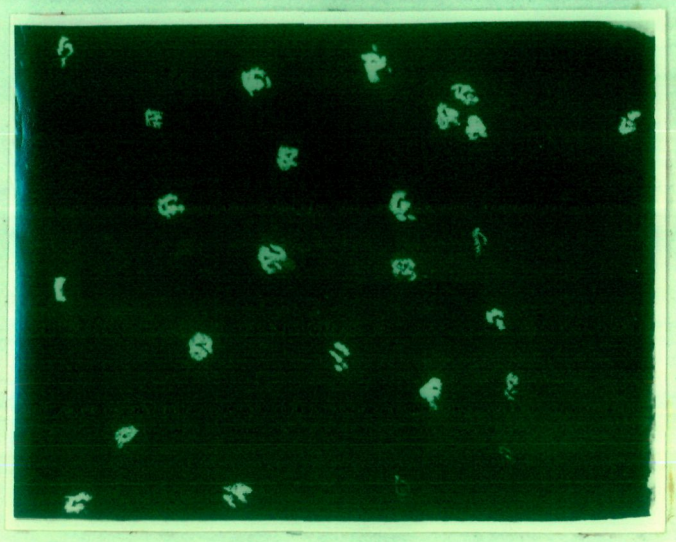


Fig. 15. Visual impression of fluorescence of P. falciparum in thick smear.

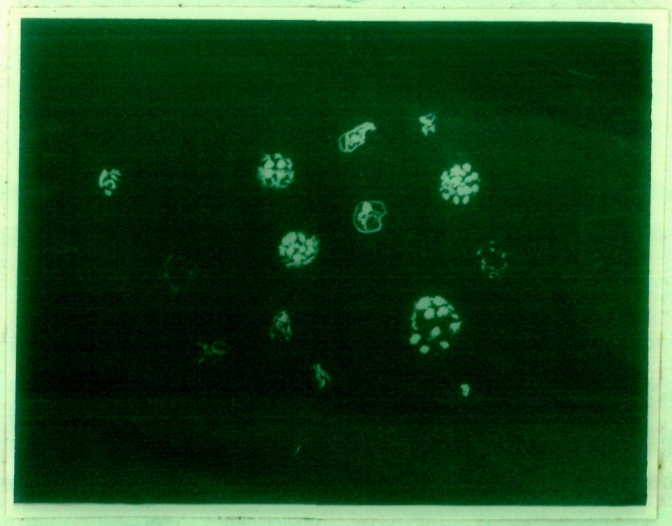


Fig. 16. Visual impression of fluorescence of schizont of P. falciparum.

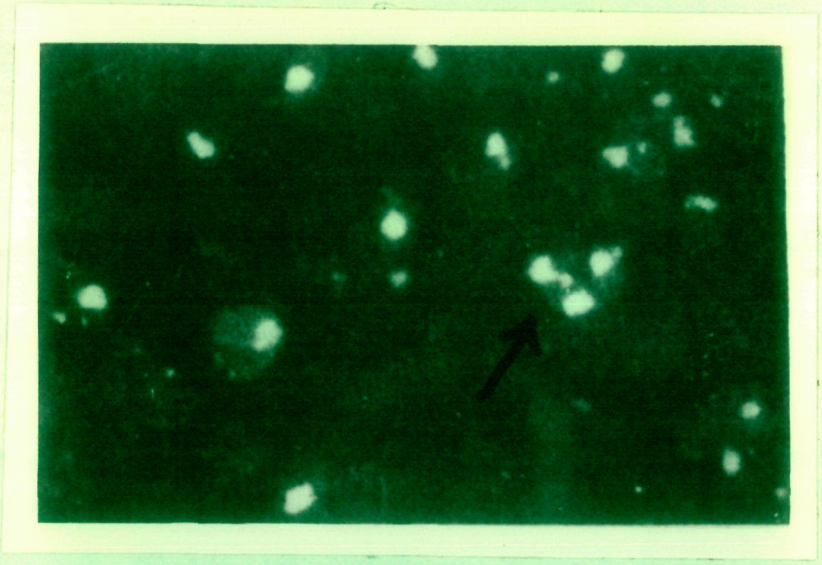


Fig. 17. Visual impression of the fluorescence of trophozoites of P. falciparum. Arrow indicates multiple infection in one RBC.

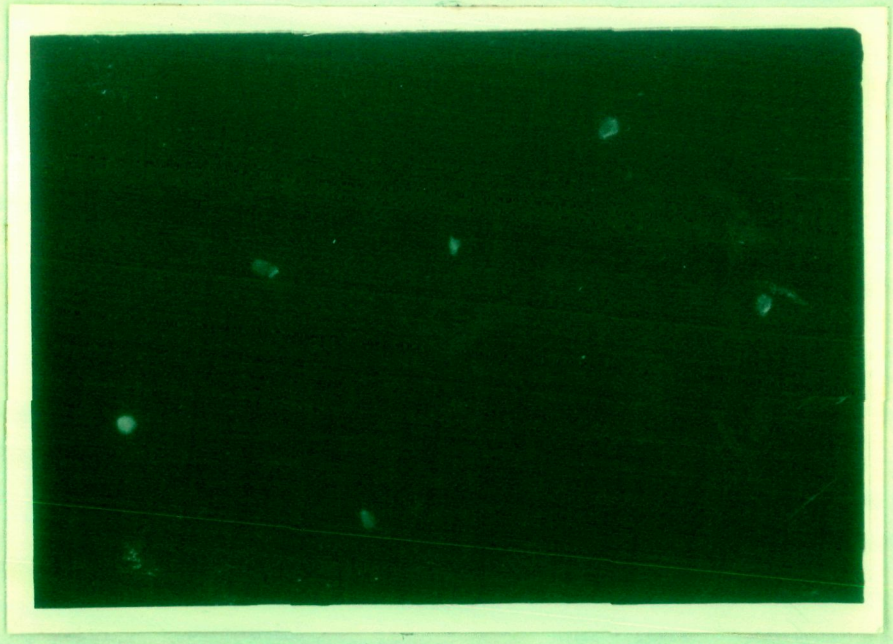


Fig. 18. Weak fluorescence of most of the rings of P. falciparum.

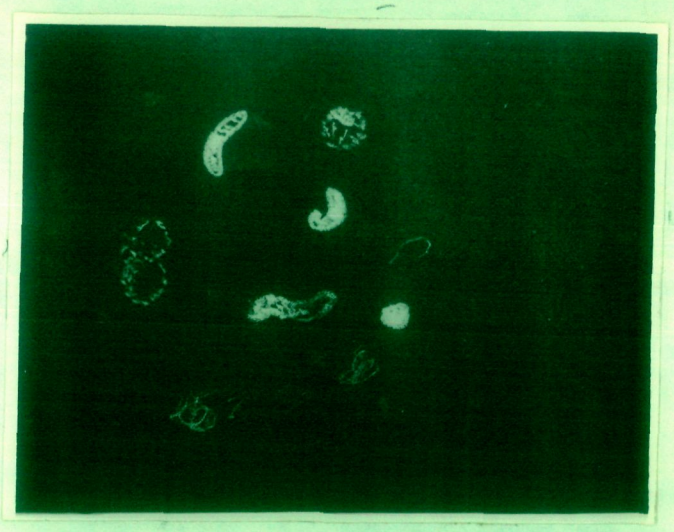


Fig. 19. Fluorescence of gametocytes of P. falciparum in a thick smear.

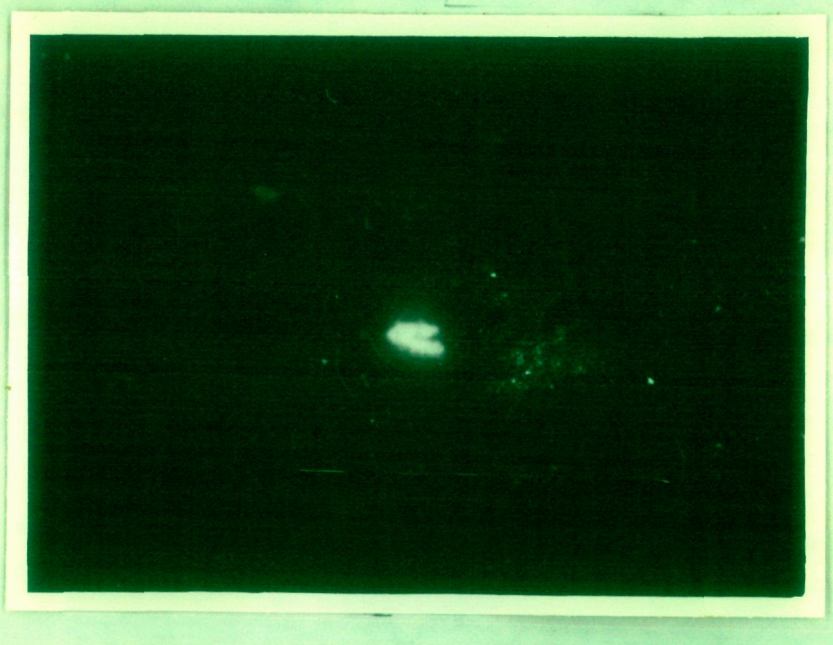


Fig. 20. Fluorescence of the nucleus of polymorphonuclear leukocyte.

infected cells were used. On the basis of fluorescence intensity the test were graded in a 1^+ to 4^+ scale. Figure 15 shows 4^+ visual impression of most of the schizonts of P. falciparum under dry high lens (40X) Figure 13-16 show 4+ fluorescence in an oil immersion (100 X) lens. In figure 17 an arrow indicates the multiple infection in one RBC. The schizonts were found as the most reactive stage in the IFA tests. Other stages also reacted but only with sera having high antibodies titres. Only weak reactions were obtained with rings, Such reactions were placed in the doubtful category, or mostly considered as negative, since visualization of fluorescence on the ring stages was difficult. There were several artifacts which sometimes, even in controls look like ring stages. Figure 18 is a photomicrograph showing weak fluorescence of ring stages.

Table 12 gives the reactivity of different stages of parasites other than schizonts. For this purpose only those sera samples were used which gave a titer of 1:128 with schizonts. Mostly old trophozoites were allowed to react with these sera at low titer level. When the infected cells were dehaemoglobinized the trophozoites were found to fluoresce more efficiently. The fluorescence in some of these sera was well defined even after raising the dilution to 2 to 4 fold. The apparent difference was only quantitiative rather than qualitative.

Gametocytes were also seen to fluoresce but there were not many samples as to arrive at a conclusion, only two such slides were used (Figure 19). False positive reactions were not uncommon. In several cases false fluorescence of normal RBC and WBC was noticed and at times it was rather difficult to decide whether the particular fluorescence was of parasitized cells. Figure 20 shows the fluorescence of the nucleus of polymorphonuclear leukocyte an example of fluorescent artifact.

4. AGREEMENT BETWEEN MICROSCOPICAL DIAGNOSIS AND INDIRECT FLUORESCENT ANTIBODY TEST IN P. falciparum and P. vivax INFECTIONS:

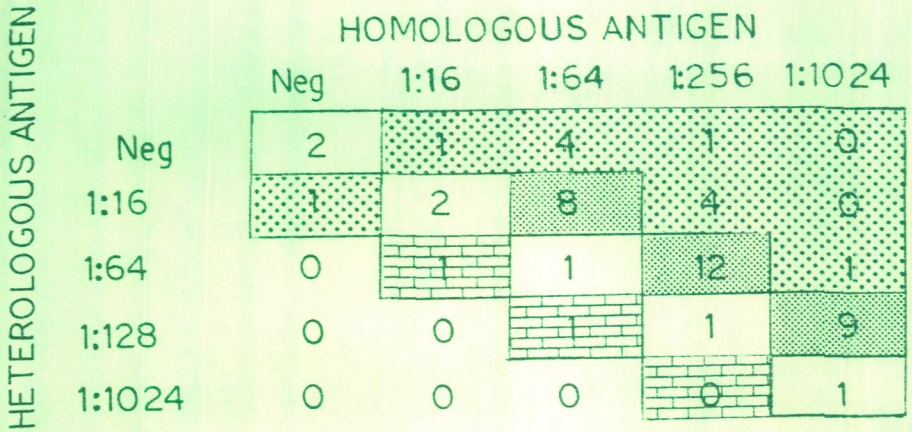
IFA tests were also done to see whether they are of some help in diagnosing the infecting species or not. For this purpose groups of patients with slide proven malaria were evaluated: Group (A) 50 patients (mostly adults) from whom a single serum sample was collected. Group (B) 40 patients from whom two or more samples were collected. In the latter group second sample was collected after the suppression of parasitaemia by chemotherapy and whenever possible few days before their discharge.

Figure 21 shows the comparison of titers with homologous and heterologous antigen smears in IFA tests. As determined by the slide examination about 30 cases were infected with P. vivax and 20 with P. falciparum. Two serum samples were negative, and 5 (10%) of the samples reacted at the same titer with both antigens. Using a four fold or greater titer difference between homologous and hetrologous antigen as criterion for species identification, over 75% of the cases were correctly diagnosed and about 6% cases were misdiagnosed i.e. the heterologous antigen gave a higher titer.

Figure 22 shows the IFA titers of serum from 40 patients from whom second samples was also obtained at the end of chemotherapy. Although there was fairely good agreement with the result based on slide diagnosis at a 4-fold or more titer, but in about 10% cases the species could not be determined by the IFA tests.

5. ENZYME LINKED IMMUNOSORBANT ASSAY FOR MALARIA USING P. falciparum ANTIGEN:

ELISA was mainly done with P. falciparum antigen obtained from the UNIVERSITY OF HAWAII and with the crude antigen preparatio



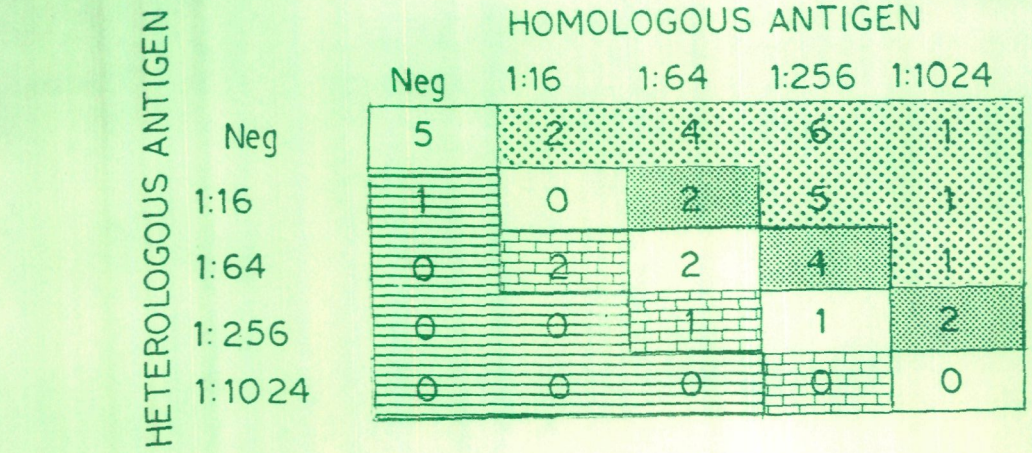
HOMOLOGOUS TITER 4 FOLD GREATER THAN HETEROLOGOUS TITER

HOMOLOGOUS AND HETEROLOGOUS TITERS ARE THE SAME

HETEROLOGOUS TITER 4 FOLD GREATER THAN HOMOLOGOUS TITER

HOMOLOGOUS TITER 16 FOLD GREATER THAN HETEROLOGOUS TITER

FIGURE 21 DIAGRAM OF LEVELS OF TITERS OF INDIRECT FLUORESCENT ANTIBODY IN SINGLE SPECIMEN OF SERUM FROM 30 CASES OF INFECTION WITH *P. vivax* AND 20 CASES OF INFECTION WITH *P. falciparum*, IDENTIFIED MICROSCOPICALLY



HOMOLOGOUS TITER 16 FOLD GREATER THAN HETEROLOGOUS TITER

HOMOLOGOUS TITER 4 FOLD GREATER THAN HETEROLOGOUS TITER

HOMOLOGOUS AND HETEROLOGOUS TITERS ARE THE SAME

HETEROLOGOUS TITER 4 FOLD GREATER THAN HOMOLOGOUS TITER

HETEROLOGOUS TITER 16 FOLD GREATER THAN HOMOLOGOUS TITER

FIGURE 22 DIAGRAM OF LEVELS OF TITERS OF INDIRECT FLUORESCENT ANTIBODY TEST IN MULTIPLE SPECIMENS OF SERUM COLLECTED FROM 40 CASES OF MALARIAL INFECTION

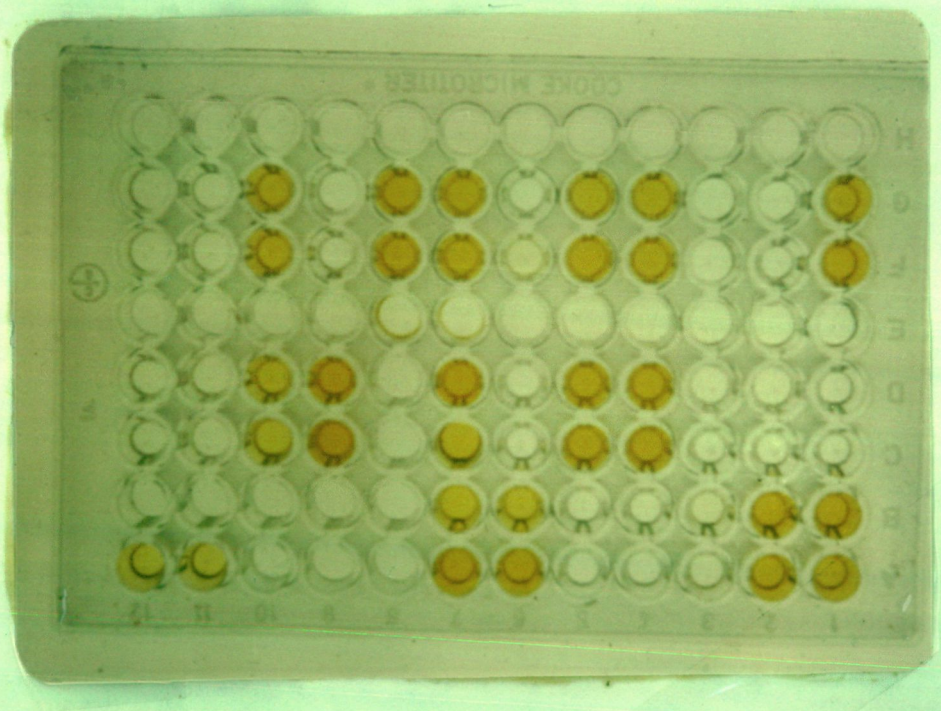


Fig. 23. Enzyme - linked Immunosorbent Assay in a polysterene microELISA plate. The colored wells are positive for specific antibodies.

from in vitro cultured parasites from this laboratory. P. vivax antigen was once used because at present there is neither any in vitro method available for producing large quantities of P. vivax antigen, nor do we have animal model (Aotus trivirgatus) to maintain the infection. Table 13 shows the results of micro-ELISA tests on polysterene plates whose wells were costed with soluble extract of P. falciparum antigen. This system proved quite adequate for the detection of antibodies in post treatment cases and for sera obtained from persons living in malarious areas. Using 57 sera for preliminary test it was observed that in both acute and post therapy samples the sensitivity rate was over 80%. Only 2 cases of acute infection and 3 cases of post therapy period gave negative results ($<$ 80). In a separate study all sera samples collected from malarious regions gave an ELISA titer of 1:80 or less. But for practical purpose we used only 1:80 or more serum titer for the infected persons. About 6 (60%) of the suspected fever cases reacted with ELISA at a titer of 80 or more. Figure

6. COMPARISON BETWEEN ELISA AND IFA TESTS FOR MALARIA:

Table 14 shows the results of 111 sera from Gabhana and 168 sera from Aligarh proper in IFA and ELISA tests. Discordance between the two tests was common in both the groups. In 22% (16.6% ELISA$^+$, IFA$^-$ and 5.4% ELISA$^-$, IFA$^+$) sera from Gabhana and in 27.4% (20.8% ELISA$^+$, IFA$^-$ and 6.6% ELISA$^-$, IFA$^+$) of the sera from Aligarh proper, a positive titer was observed in only one of the two tests employed. Majority of the cases were either positive or negative by either of the two tests, showing an acceptable correlation in the specificity of the two tests.

Table 15 shows the detection rate of the IFA and ELISA tests for malarial antibody. Agreement was best in the first two groups,

TABLE 13

Enzyme - linked Immunosorbant Assay (ELISA) using whole Anti-immunoglobulin conjugate

Sera group	No. of sample examined.	No. of sera with an ELISA titer of					
		80	1:80	1:160	1:320	1:640	1:1280
Acute infection	25	2	6	5	8	4	-
Post-treatment	22	3	2	0	4	4	8
Suspected fever cases.	10	4	3	2	0	1	-
No. of replicates		2	2	2	2	2	2

TABLE 14

Correlation between enzyme-linked immunosorbant assay (ELISA) and Indirect Fluorescent Antibody (IFA) tests in 111 sera from Gabhana and 168 from Aligarh proper.

Results	Gabhana sera %	Aligarh sera %
$ELISA^+$ IFA^+	54.4	46.2
$ELISA^+$ IFA^-	16.6	20.8
$ELISA^-$ IFA^+	5.4	6.6
$ELISA^-$ IFA^-	23.6	26.4
Total No. examined.	111	168

TABLE 15

Comparison between antimalarial positive and negative IFA and ELISA tests in sera after the onset of malarial symptoms*

Interval since	IFA	+	-	+	-	Sera
Onset of symptons	ELISA	+	-	-	+	Nos.
0-14 days		46(79.3)	4(6.9)	6(10.3)	2(3.5)	58
15-30 days		58(92.1)	1(1.6)	4(6.3)	0(0)	63
30-60 days or more		12(31.6)	9(23.6)	0(0)	17(44.7)	38

* percents are shown in parenthesis.

showin an average of 93.7% between 15-30 days (2 weeks) after the onset of illness. The serum drawn one month after the oneset of symptoms showed some disagreement particularly in samples which were ELISA positive but IFA negative. Antibody is apparently detected for a longer period of time in the ELISA test than by the IFA test procedure .

7. THIN LAYER IMMUNOASSAY FOR DETECTION OF ANTI-MALARIAL ANTIBODIES:

Figure 24-25 shows two photographs of DIG-TIA plates, both coated with soluble P. falciparum antigen. The antigen coated plates were used for testing sera samples obtained from malaria patients. Visualization immediately after completion of diffusion revealed weakly positive results for some of the sera samples. The plates, when floaded with anti IgG or Anti IgM, showed a reinforcement of these reactions. The doubtful reactions, having small areas with slight wettability (indicated by arrows in fig 25) were considered as negative. These reaction were observed in some sera samples obtained from U.S. blood donors. Based on the results from 34 sera samples, the limit between positive and negative TIA reactions was determined at about 50 mm^2 (zone diameter $\simeq$ 8 mm). An area of less than 50 mm^2 was considered negative even with sera positive by any other serological tests.

Results obtained by TIA and other serological tests such as IFA and ELISA are summarized in table 16. There was a fairly good agreement between the results obtained by IFA, ELISA and TIA. Thus a total of 20 of the 34 sera were positive by all the three tests mentioned, while only 5 were positive by only one or two of the tests employed. There were also 5 sera samples which gave negative TIA results but were positive in the other two tests. Only one sample gave a false

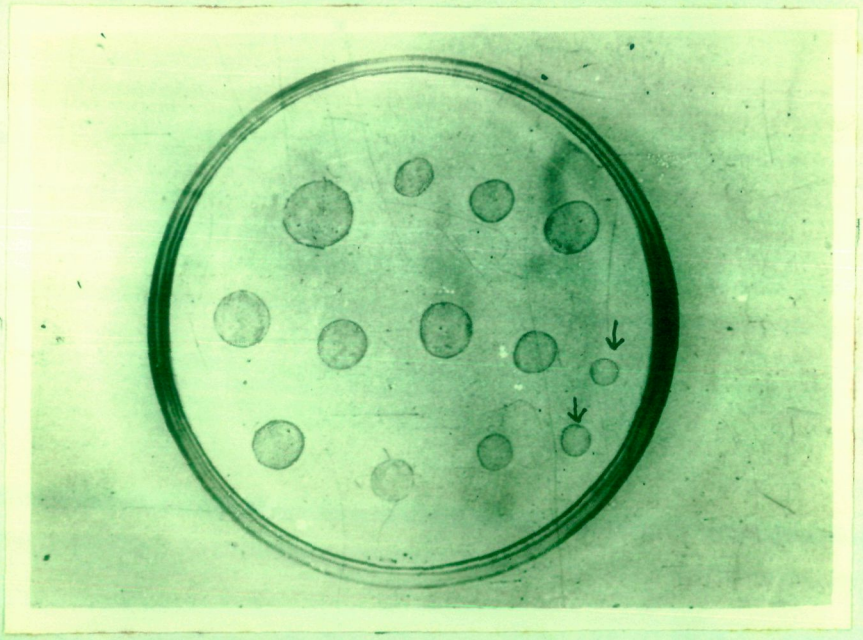


Fig. 24. Photograph of a DIG-TIA plate showing positive reaction sites as visualized immediately after removal of agar gel.

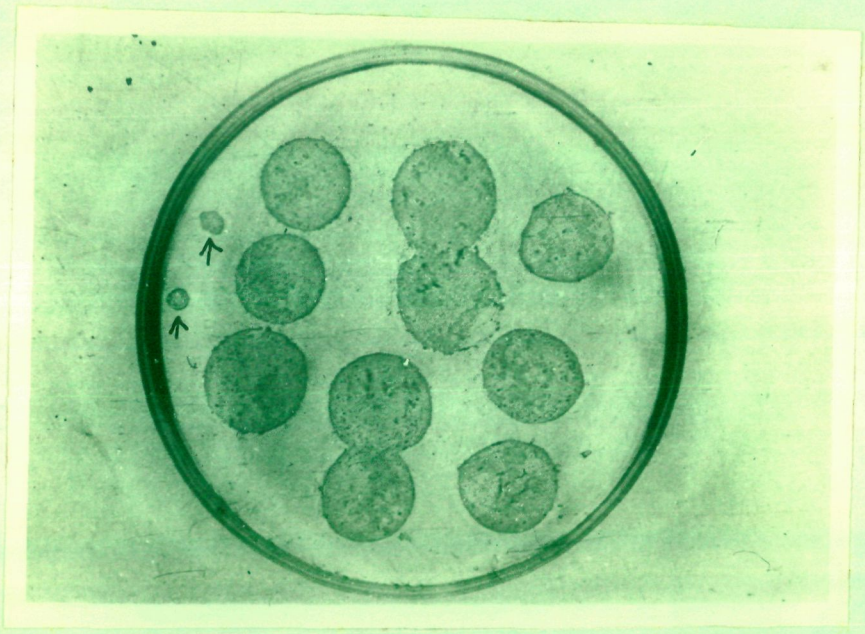


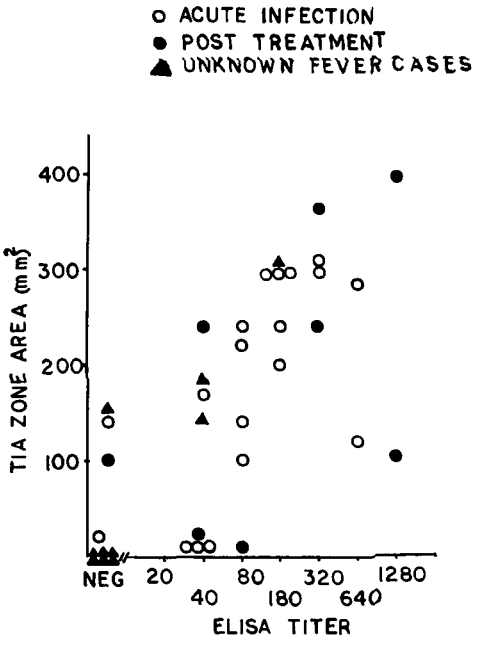
Fig. 25. Photograph of a DIG-TIA plate showing positive reaction sites after reinforcement with anti-IgG.

TABLE 16

Comparison of thin layer Immunoassay with the serum reactivity in different antibody assays against Plamodium falciparum.

Test Results	Patients				Mean TIA area(mm^2)
	Acute infection	Post treatment	Unknown fever cases	Total No.	
$ELISA^+$ IFA^+ TIA^+	15	4	1	20	255
$ELISA^+$ IFA^- TIA^+	1	0	1	2	115
$ELISA^-$ IFA^+ TIA^+	1	0	0	1	120
$ELISA^-$ IFA^- TIA^+	0	0	1	1	70
$ELISA^+$ IFA^+ TIA^-	3	2	0	5	50
$ELISA^-$ IFA^- TIA^-	1	0	4	5	< 50
Total:	21	6	7	34	

ELISA - Enzyme -linked Immunosorbant assay ; IFA - Indirect Immunofluorescent Antibody test ; TIA - Thin layer Immunoassay.



COMPARISON OF TIA TEST AREAS WITH ELISA TITERS IN 34 SERA SAMPLES

Figure 26.

positive result. Sera collected from infected persons and post treatment cases were both found reactive with in TIA tests.

Figure 26 illustrates the relationship between a TIA reaction area and the ELISA titer value. No distinct correlation was observed between these two parameters. The TIA test result were not always reproducible. The difference was quantitative rather than qualitative and most of the TIA positive sera were reactive with both IFA and ELISA tests.

CHAPTER V

DISCUSSIONS

DISCUSSION

Malaria is one of the world's most severe health problems for inhabitants of tropical and subtropical areas. The morbidity and mortality resulting from this infectious disease severely hamper the economic progress of these regions. In many such regions, malaria may not be a direct cause of significant morbidity andmortality. But it certainly acts as a "great umbrella" for making a large number of immune compromised individuals easily susceptible to other more life threatening diseases (Down, 1975).

Malaria has returned to Asia in epedemic, proportions, bringing in its wake misery to millions of its inhabitants. It has significantly contributed in undermining already poor economies and calling into question the judgement of those who once confidently predicted that the disease was on the way out. At the time of Independence in 1947, it was estimated that India had some 75 million malaria sufferers with 800,000 deaths in a year. With the start of National Malaria Eradication programme in 1958, a rapid fall in morbidity and mortality was achieved and by 1965 the eradication of this scourage seemed to be almost within grasp. In that year it was estimated that there are not more than 100,000 active cases. However, at what now appears to have been a crucial stage the campaign faltered owing to a variety of reasons, the resurgence of malaria came into focus in 1966 with an ever increasing number of cases. The number of cases increased fairly rapidly and steadily to over 5 million recorded in 1975 and 1976, with 10 million in the first nine months of 1977.

Malaria is caused by the parasits of genus Plasmodium infecting a wide range of vertebrate hosts. Of , the four species of plasmodia

(P. falciparum, P. vivax, P. malariae and P. ovale) infecting human beings, P. falciparum produces the most severe form of the disease, often causing death to the infected host. The clinical picture presented by these species is highly variable. It is usually necessary to develop altermate laboratory procedures for use as an adjunct to microscopic examination in which parasites may not always be detectable. The demonstration of parasites in the host blood is considered diagnostic, though negative findings do not always exclude the infections altogether. This is specially true in cases of early infections when the parasite density is low or where the blood picture is some what obliterated due to chemotherapy. For making a defferential diagnosis it is often necessary to rely on indirect immunodiagnostic procedures, in addition to clinical and microscopic observations. The immunoassays has gained importance in recent years, as they not only provide a useful means of identifying infected persons but are also used as a valuable tool for epedemiological surveys.

The provision of suitable malarial antigen has always been a limiting factor for the immunoassays. But the availability of Aotus trivirgatus griseimembra (the owl monkey) as a laboratory animal model for human malarial species (Young et al., 1966; Geiman and Meager, 1967; Geiman and Siddiqui, 1969) and the development of long term in vitro culture of P. falciparum (Trager and Jensen, 1976; Siddiqui et al., 1974) have to a certain extent solved the problem of non availability of malarial antigens.

Since 1979 we are using a static flask culture system for growing P. falciparum in vitro since then we have cultured several strains of P. falciparum for an extended period of time. The cultures

were held in 125-ml erlenmyer flasks. A total of 10 ml volume contained 10% red cell suspension (5% packed cells), 10% human serum and 90% RPMI-1640 medium supplemented with 25 mm HEPES buffer. Results presented here show that RPMI-1640 medium with 25 mm HEPES buffer supports the growth of P. falciparum in vitro. In all the cultures the parasites were seen to go through their schizogonous cycle with a definite reinvasion at the end of 48 hours of incubation. However, the growth pattern of different strain is variable. The parasitaemia on day o was monitered at approximately 1-2% which was increased to 5.6-11.9% at the end of day 4. Thus the overall increase in parasite count averaged 4-5 fold with a net increase of 10-fold per week. These findings are encouraging, especially in view of the fact that at each transfer there is some loss of erythrocytes. These results are in conformity with those of Trager and Jensen (1976) and Siddiqui et al., (1974). General attempts were initially made (Anderson, 1953; Bass and John, 1912; Trager 1947 and 1971; Phillips et al, 1972 and siddiqui et al., 1970) to grow malarial parasites for an extended period of time with varying degrees of success. Trager (1947) obtained significant multiplication of P. lophurae by serial sub-culture over an 8 day period. Anderson (1953) working with P. gallinaceum found continuous growth and multiplication by successive subculture over a period of 2 weeks. The favourable effect of erythrocyte extracts was noteworthy in both of these studies. Earlier experiments, (Trager 1971) using a technique in which a slower flow of medium was maintained over the settled layer of cells, was successful in growing P. falciparum and P. coatneyi through two cycles of schizogonous development (i.e. a toal of 4 days) with no net increase in the total number of parasites. In our experiments when the medium was changed every 24 hr, an increase in the number of parasites was obtained upto the third schizogonous cycle, and even at the end of the fourth cycle the parasite number remained at about the level of the second generation. In 1972 Philips et al.,

were able to sub-culture P. falciparum and P. knowlesi through almost three cycles in vitro with a marked increase in number during the first sub culture, with the number being maintained only during the second sub-culture.

Trager and Jensen (1976) were the first to report the successful continuous in vitro culture of FVO strain of P. falciparum. In the same laboratory, a simplified technique, the petri dish-candle Jar, was developed for continuous cultivation of P. falciparum (Jensen, 1979). Starting with a parasitaemia of 0.1% Trager and Jensen (1980) were able to obtain a 20-50 fold increase to about 2-5% parasitaemia after 96 hour. Our static flask culture system differs from Trager and Jensen's petri dish-candle Jar method in that we provide a continuous flow of 2% 0_2, 8% Co_2 and 90% N_2. In candle-Jar system the gas phase is estimated to be 2-3% Co_2 and 14-15% 0_2. In terms of medium and serum concentration our system is the same. Instead of 8% red cell suspension and 0.1% parasitaemia we use 5% red cell suspension with 1-2% starting parasitaemia. A major advantage of this flask system over the petri dish-candle jar method is that it can be easily adapted to a wide range of convenient volumes. (Table 3). This adaptibility is obviously lacking in the petri dish candle Jar system as there is a limit to the size of dessicator or other similar container that can be used and to the size of a petri dish that can be easily handled.

From the results reported in table 6, it is clear that 10% human serum supports the growth of P. falciparum pretty well. In flasks containing less than 10% serum the extent of multiplication was much less though the growth and morphology of the parasites were not very unsatisfactory. The serum conc. more than 10% was also found to be inhibitory for few isolates. Trager (1976) and Siddiqui (1979) reported that serum concentration above 15% have

negative effects on growth. Siddiqui (1979) was able to grow adapted cell line in 2.5% serum but routinely used 10% serum. Several investigators reported that the ability of serum to support growth varies only marginally. A strict check on the type of serum to be used is essential, especially in areas where malaria is endemic. Because immune serum and serum from individuals taking antimalarial drugs are detrimental to parasite growth and multiplication. Attempts were made to culture P. falciparum in sera samples obtained from Indian Blood Donors (Table 7). The parasites in cultures with Indian sera samples could complete one schizogonous cycle but were unable to envade fresh red blood cells. This effect was not noticeable in cultures containing sera from U.S. blood donors. This can be attributed to the presence of antibodies in Indian sera samples (RIZVI and Ahmad, 1982). Also indiscriminate use of antimalarial drugs, antibiotics and antiamoebic drugs containing chloroquine may also contribute for the inhibitory effect of Indian sera in malarial cultures.

Recent work has indicated that the susceptibility of the host cell is an important factor. This effect was also noticeable in our experiments and comparatively better growth was obtained in A^+ and 0^+ erythrocytes. However, other cell types did support the growth of P. falciparum (Figure III). However, we were not certain as to the actual relative susceptibility of the various cell types. Trager (1971) found that human group A and not human group B or AB erythrocytes, agglutinates with Aotus erythrocytes. Experiments on the cultivation of P. falciparum from Aotus monkeys resulted in limited multiplication during a single in vitro cycle (Siddiqui et al., 1970). Greater multiplication resulted by mixing parasitezed blood from Aotus monkeys with human erythrocytes, Trigg (1975) using a subculture technique showed that P. falciparum originally grown in vitro in Aotus erythrocytes would invade human group O cells to a greater extent than Aotus

erythrocytes. Reports so far available indicate the suitability of all ABO cells. The significance of this is not clear since no one has attempted to culture P. falciparum serially in non-human erythrocytes. But it seems quite likely that human erythrocytes maintain their viability and/or susceptibility to infection in vitro better than non-human erythrocytes. The most interresting report is given by Trager and Jensen (1976) that the successful cultivation of P. falciparum can be done in out dated blood from blood bank.

Attempts to culture P. vivax have been unsuccessful in our laboratory, a result not very different from several other laboratories of the world. From table 8 it is clear that the parasites were seen to complete their schizogonous cycle but their number remained constant. On average, only one merozoite from each schizont succeeded in invading fresh red cells. The results are slightly better when P. vivax was cultured in waymouth's MB-752/1 medium. The results are not comparable with those of P. falciparum where the same technique was used. Several attempts to culture P. vivax were also made by Bass and John, 1912; Lovinder, 1913; Thompson and Thompson, 1913; Zuman, 1913; Row, 1917, 1929, Geiman et al., 1966 and Siddiqui, 1979. In all these studies in vitro growth of P. vivax was limited upto the schizont stage. It appears that in vitro growth requirements of P. vivax are somewhat more complex. P. vivax preferabily invades only reticulocytes. Reticulocytes have substantially more enzyme activity than do erythrocytes. Hexokinase is one of the enzymes in which the activity declines most rapidly during red cell aging. Our inability to provide adequate cell populations of reticulocytes in cultures appeared to be one

of the major obstacles in culturing P. vivax. Also the glucose consumption by P. vivax is at least 3-4 times higher than that by P. falciparum (Geiman, et al., 1966), resulting in greater production of lactic acid with a decrease in the pH of the culture. Therefore, the maintenance of pH through the use of various buffers (Siddiqui, 1973) must be a prime requisite for successful cultivation of P. vivax. At present, there are a few hints, but no definite procedure is available for continuous cultivation of P. vivax. Much more extensive work is required before the long-awaited goal for continuous culture of P. vivax can be accomplished.

The fluorescent antibody test was initially introduced by Tobie and coatney (1961) and voller and Bray (1962). In the past few years, a number of workers have made important contributions with this method (Kuvin et al., 1962 voller 1968). The indirect fluorescent antibody test is nowadays most widely used for the diagnosis of malaria at the present time. Reports by Mc Gregor et al., (1963) in Gambia on the use of IFA tests for measure the status of immunity in a population residing in an endemic areas have shown that serology may be used to good advantage in the epidemiologic assessment of malaria endemicity.

We have used a washed cell-thick smear antigen in all our IFA tests. Results of IFA using this method (Tables 9, 10, 11) show that this test appears to have good reproductibility, sensitivity and specificity. Very small percentage of sera samples gives false positive results. Since the sera samples were collected from malaria endemic areas, the possibility of persisting anti-bodies can not be altogether excluded. Keeping this in mind we have considered 1:16 as the cut off titer between true positive and

negative results. These results are in good conformity with those of Tobie and Coatney, 1961, voller, 1962; collins et al., 1966; Diggs and Sadun, 1965; Wilson, 1970 and sulzer et al., 1973 who all used thick smear antigen preparations. Sulzer et al., (1969) made a major practical contribution when they showed that washed infected erythrocytes could be used as thick blood smear antigen. This permitted the use of much lower parasitaemias. In fact multiple thick blood smear spots could be made on each slide, thus allowing many more samples to be tested (voller and O'Neill, 1971). In our studies, most of the false positive results were seen when antigen slides were made directly from the patients. That's why we have always used washed cell thick smear antigen prepared from in vitro cultured P. falciparum. By this the appearance of false positive results could be reduced. Washing the parasitized cells removes soluble serum componants, especially gamma globulin that may contain malaria antibodies rendering the test unreliable. The use of in vitro cultured parasites solves the problem of scarcity of mature stages of the parasites which in case of P. falciparum are not found in the peripheral circulation of the infected persons. The antigen slides prepared from in vitro cultured parasites can also be stored for several years at -70°C. In our studies too, the stored slides gave good results. The multispecies malaria slides containing P. vivax, P. falciparum and P. malariae of Sulzer et al., (1973) provide a suitable antigen for maximum sensitivity in detecting antibody against the three main human malaria infections. Unfortunately difficulties in its production means that its use will remain restricted to a few centres only.

The IFA response was variable in different age group sera in these studies. But this test procedure was useful to detect antibodies in children as well as adults inhabiting malaria endemic areas (Table 10). Results show that antibodies levels

were low in sera obtained from 0-14 year age groups and were found to increase considerably in age groups of 15-more. The finding of voller and Bray (1962) supports our results. They demonstrated that antibody could be detected in virtually all the inhabitants of a malaria hyperendemic area of Liberia. They also showed that the antibody levels tend to increase with age. McGregor and Colleagues (1965) made a much more detailed study of the malaria IFA response in Gambia. Children were born with high levels of antibody which was found to decline over the first few weeks of life. The antibody levels graduately increased through childhood to adult life.

Homologous human plasmodia were best used as antigen in IFA tests. This often poses some difficulties in obtaining good antigen source. The mature schizonts, which were known to be most reactive (Targett, 1970) do not occur in human peripheral circulation in P. falciparum infections. Details as regards to the usefulness of other froms of parasites are lacking. We have tried to observe the reactivity of rings, trophozoites and gametocytes obtained from in vitro culture pools. In all these tests, the schizonts reacted best in IFA with a seropositivity rate of over 90% in schizont antigen models. Other stages reacted with high titer sera samples. Results in table 12 clearly indicate that the reactivity of trophozoite could be increased by dehaemoglobinizing the infected cells. By using such preparations the dilutions of sera could be raised to 2-3 fold in some cases. This can be attributed to the intactness of plasma membrane of the red cells containing rings and trophozoite stages. The plasma membrane of red cells containing these stages act as more efficient barriers to prevent the complexing of antibodies, while the situation seems to be reversing with the maturing of parasites.

The weak fluorescence of trophozoite infected cells could also be attribute to the presence of soluble antigen in caveola-vescicle complex along the membrane of infected cells (Aikawa et al., 1975). The difference in reactivity was found to be only quantitative and appeared to play a minor role in the over all usefulness of the test. False fluorescence of non-infected erythrocytes may be due to the presence of antibodies against ABO blood group system. We have rectified this difficulty by using parasites grown in vitro in O^+ cell types. Thus with careful precision and procedural manipulations one can get good reproducibility, sensitivity and specificity with the thick smear antigen from in vitro cultures as well as human subjects.

Because of the varied nature of clinical picture presented by four human plasmodial species, it is necessary some times to use indirect immunological methods for use as an adjunct to microscopical examination. Because many a times, the parasites may not be detectable on the slide due to a very low parasitaemia. With the introduction of IFA tests, there has been a reniewed interest in the use of this serodiagnostic tool identifying and making differential diagnosis of infecting species (Gleason et al., 1971). We have also used IFA tests for the identification of species and to compare the results with those of microscopical examination. The data presented in figures 21 and 22 indicate that determination of the infecting Plasmodium species by means of indirect fluorescent antibody tests is quite feasible. A four fold difference in titer levels between homologous and heterologous antigens accurately indicated the infecting species in atleast 75% of the sample tested; only about 6% samples were misdlagnosed. Significantly good results were obtained when serum samples were drawn after the chemotherapy (Figure 22). These results are in good conformity with those of Gleason et al., (1971) and

Wilson (1972). Voller (1962) reported on IFA cross-reactions between the different primate malarias studied, and Tobie et al., (1961) expressed this quantitatively. They found that the titers were always highest in the homologous parasite antiserum system. This difference has been confirmed for many primate and human malarias (Collins et al., 1966 a, b, Diggs and Sadun 1965; Wilson et al., 1970). In some of our experiments the titer level of the heterologous antigen was the same or greater than that of the homologous antigen. These may be accounted for by residual effects of previous attacks of another species, since all the sera samples were collected from malaria endemic areas.

The enzyme-linked immunosorbant assay (ELISA) was developed as an alternative to radioimmunoassay (RIA) for the detection of soluble antigen and antibodies (Engvall and Perlman, 1971; 1972). Since then ELISA has been used for many parasitic diseases including malaria. Voller et al., (1975) utilized P. knowlesi in their ELISA tests for the detection of antimalarial antibodies in Iran and Tanzania. Later on Voller 1976 found the usefulness of P. falciparum derived from Aotus monkeys. We have utilized P. falciparum antigen obtained from in vitro culture for use in ELISA tests. Results in table 13 indicates the usefulness sensitivity and specificity of ELISA tests. However, ELISA is of value in detecting antibodies more efficiently in the post treatment period. False positive results were not uncommon. The results suggest that P. falciparum obtained from in vitro culture appear to be an excellent source of antigen for the micro ELISA tests for malaria. The ELISA test itself is a promising serologic test for malaria in a large population, or in individuals. Reproducibility of the titers on a test-to-test basis is excellent (Table 13). 4 positive sera were tested several times and all

titers were replicated with in plus or minus one (four fold) dilution. Similar results were obtained by spencer et al., (1979) and others in several laboratories.

A comparison of the reactivity of ELISA and IFA tests is given in table 14 and 15. Results obtained by ELISA test using P. falciparum obtained from in vitro culture (as antigen) and the IFA test for P. falciparum antibody did not correlate well. An appreciable proportion of the specimens were positive by one test but not by both. Thus even in situations where the serologic profile given by the two tests is the same, samples seropositive by one test were not necessarily positive in the other test. This suggests the detection of some shared and some dissimilar antibodies by the two tests. In some cases, neither test was suitable to detect malaria antibodies from patients who were slide diagnosed. The four serum samples in 0 to 14 - day category (Table 15), undetected by both test, may have been drawn too early in the attack before the antibody reaching a detectable level. In all studies we found that IFA test tends to detect antibody earlier in the disease than the ELISA. Perhaps non-precipitating antibody is produced initially, which could be detected by IFA but not by ELISA. Non precipitating antibody might also participate in the ELISA tests in the presence of precipitating antibody for inhibiting or blocking the reaction (Voller, personal communication). At the moment there is not sufficient data to determine the causes of the ELISA negative tests in some proven infections. Further assessments of the ELISA tests are needed to determine the obtimum condition under which positive responses can be expected and also continue to correlate the ELISA results with those of IFA. Although the ELISA methodology has considerable promise for future use in the field, but its widespread adoption must follow a better understanding of its limitations.

Thin layer immunoassay is a recently developed technique (Elwing et al., 1876, 1977) which has been applied to the sero diagnosis of various types of parasitic infections Nilsson et al., 1980) , as well as of Herpes simplex viral infections (Jeansson et al., 1979). Because of its technical simplicity and high capacity, a need was felt to use this technique for the detection of antimalarial antibodies. Results of TIA using in vitro cultured P. falciparum (table 16) shows very good sensitivity and specificity of this test. This procedure was found to be technically simple and of low cost as reported by Nilsson et al., 1980. The wettability of TIA zone contrasted well with the background, thus allowing an easy and rapid interpretation of the results. Also a number of sera could be screened on one plate (Figure 24). Another advantage is the possibility of using in vitro cultured parasites as antigen in TIA tests. Although the antigen coated was the crude extract but this gave us good results. Since this was a priliminary study that's why we did not try purified antigen preparations. Five of the specimens tested did not react in TIA tests though they were positive by IFA and ELISA tests. This can be attributed to the presence of extraneous proteins (Cellular componants) in crude antigen preparation which may decrease the sensitivity of the assay procedure. The purification of antigen can certainly be expected to increase its sensitivity. It may also be possible that TIA would detect some different class of antibodies dissimilar to that detected by IFA or ELISA tests. This may be one of the reasons for disagreement of results in TIA reaction areas and ELISA test titers. No definite correlation was obtained in these two tests (Figure 26).

In addition to its technical simplicity and high capacity, TIA has the advantage over IFA and ELISA test in that the diffusion-in-gel procedure makes the time consuming preparation of serial

dilutions unnecessary. Also no specialized reagents are required, except the antigen coated plates. However, TIA test was not always reproducible. The difference was quantitative having had little effect on the overall positivity of the test sample. Most of the TIA positive sera were reactive either in IFA or ELISA tests or in both. The present study did not allow any difinite conclusion because of the small number of sera tested. More elaborate studies are required on the sequential sera samples obtained from clinically defined patients to substantiate and define the use of this new technique in serodiagnosis of malaria vis-a-vis its usefulness with IFA and ELISA test result. Our results with limited number of sera showed that TIA procedure can be used as an adjunct to IFA and ELISA tests.

CONCLUSION

In the foregoing study attempts were made to evaluate some of the immunological techniques which measure antibody responses during plasmodial infections. The identification and measurement of malaria antibodies is an imperative for identifying the infected persons from those who may constitute a mixed population of the immune and more susceptible hosts. For such epidemiological surveys. Indirect Fluorescent Antibody (IFA) test is one of the most widely used laboratory procedure described in the recent past.

With washed-cell thick smear antigen the IFA tests for Malaria are almost as reproducible, sensitive and specific as any other reliable laboratory diagnosis. The infecting species of malaria can be readily identified by means of this serological method. Specially in cases where malaria is suspected but definitive diagnosis can not be made by means of blood films, the IFA tests are usually more useful. For maximum diagnostic specificity and sensitivity, homologous plasmodial species antigen should be used. In vitro cultivation of human plasmodia provides an excellent source of antigen for IFA tests. The results of IFA tests are comparable with those of other serological tests such as ELISA, TIA etc. The IFA is of particular interest in detecting antibodies in acutely infected persons while ELISA appears to be more reliable in detecting late antibodies.

The presence of circulating antibody in a patient and his functional immunity are of course two different aspects. It must be stressed that detectable antibodies do not necessarily indicate ones immune status, or reflect the measure of protective immunity of an individual or a population. In some instances antibody levels correlate with the degree of protection, but this may not always be universally true.

As yet, there is no single immunologic test for malaria which would directly and invariably reflect body's operational immune defences against such a well adapted parasite as human malaria.

CHAPTER VI

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APPENDIX

LIST OF PRESENTATIONS

1. S. Farrukh Sultan Rizvi and Sohail Ahmad. Indirect Fluorescent Antibody (IFA) Test for Malaria Using Different Stages of Parasites as Antigen. Paper presented at 50th Annual Conference of the Society of Biological Chemists (India) held at Baroda, Nov. 18-20, 1981.

2. S. Farrukh Sultan Rizvi, Salahuddin and Sohail Ahmad. In vitro Cultivation of Erythrocytic Stages of P. falciparum. Presented at the National Conference of Protozoology held at Aurangabad, Dec. 9-10, 1981.

3. S. Farrukh Sultan Rizvi and Sohail Ahmad. In vitro cultivation of P. falciparum using sera from Indian Blood Donors. Presented at 4th National Congress of Parasitology held at Aligarh. March 1982.

4. S. Farrukh Sultan Rizvi, Sohail Ahmad and M. Ekram Siddiqui. Detection of Antimalarial Antibodies in Human Sera samples by means of Thin Layer Immunoassay(TIA) technique. Presented at 51st Annual Conference of the Society of Biological Chemists (India) held at Chandigarh, Nov. 18-20, 1982.